

# **Cerebral Magnetic Resonance Spectroscopy Biomarkers and Outcome in Perinatal Asphyxia**

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## **Declaration**

I, Manigandan Chandrasekaran, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

Date:



## **Abstract**

**Background:** Neonatal encephalopathy is a common clinical condition affecting 2-3 per 1,000 neonates in the developed world. 10-15% of cases will die in the neonatal period. Therapeutic hypothermia has become the standard of care in the developed world for infants with moderate to severe neonatal encephalopathy only recently, after 2 decades of experimental and clinical studies. Approximately half the infants who receive therapeutic hypothermia still have an abnormal outcome. Research is now being focused on pre-clinical and Phase II clinical studies of drugs, which act synergistically or additively with therapeutic hypothermia. Magnetic resonance spectroscopy (MRS) techniques provide translational biomarkers, which may be used to speed up the development of safe and effective neuroprotective interventions. The precise relation between MRS and brain histology is unknown and it is unclear whether cooling itself alters the prognostic efficacy of MRS.

**Aim:** (i) To explore the relation between MRS biomarkers and the degree of brain pathology observed in our pre-clinical piglet model of perinatal asphyxia; (ii) To assess the predictive value of MRS biomarkers in infants with neonatal encephalopathy

**Methods:** In our piglet study, data from 2 large piglet asphyxia studies investigating neuroprotective agents were analysed. 1. The first was a study of Xenon-augmented hypothermia. Following transient hypoxia-ischemia, 36 piglets were randomised into 4 groups (each n=9), with intervention from 2-26 h: Group (i) normothermia (38.5°C); Group (ii)

normothermia (38.5°C) + 24 h 50% inhaled xenon; Group (iii) 24 h therapeutic hypothermia (rectal temperature ( $T_{\text{rectal}}$ ) 33.5°C) or Group (iv) 24 h 50% inhaled xenon +24 h therapeutic hypothermia ( $T_{\text{rectal}}$  33.5°C). 2. The second was a study investigating Amiloride as a neuroprotective agent. Following transient hypoxia-ischemia, 18 male piglets (<24 h of age) were randomized to 2 groups (each n=9) (1) normothermia; or (2) 2.5mg/kg of methyl isobutyl amiloride (MIA) at 10 minutes after resuscitation and 8 hourly thereafter.

Both studies were performed on a Bruker 4.7 Tesla MR system. Following resuscitation after hypoxia-ischemia, proton ( $^1\text{H}$ ) magnetic resonance (MR) spectra were acquired with repetition time (TR) 5 sec, 128 summed transients, and echo times (TE) 25 ms, 144 ms, and 288 ms. (we measured the following peak area ratios: Lactate/N acetyl aspartate (Lac/NAA) and Lactate/Creatine (Lac/Cr) in both white matter and thalamus). Phosphorus ( $^{31}\text{P}$ ) MR spectra were acquired from whole brain using single-pulse acquire with TR 10 s (we measured inorganic phosphate (Pi)/exchangeable phosphate pool (EPP = Pi + PCr + (2 $\gamma$  +  $\beta$ )-NTP), NTP/EPP). Immunohistochemistry was performed on brain sections for activated Caspase 3, TUNEL positive cells and Iba I (activated microglia) to quantify cell death and microglial activation.

For the clinical study, we assessed 45 infants (median gestational age – 40 weeks) with moderate to severe neonatal encephalopathy admitted over a 3 year period to the neonatal unit at UCH. Their neurodevelopmental outcome was assessed at 18 months.

**Results: (i) Experimental Study - Early Biomarkers** Early biomarkers (acquired between 2 and 4 hours after hypoxia-ischemia) in particular

thalamic Lac/NAA, predicted the 1H MRS area under the curve values from 0-48h and quantitative immunohistochemistry at 48 hours. **Late Biomarkers:** WM Lac/Cr at 40-48h after HI demonstrated the highest positive correlation with TUNEL positive cell death ( $R^2 0.98$ ,  $p = 0.01$ ) and NTP/EPP with microglial ramification ( $R^2 0.68$ ,  $p = 0.006$ ); this correlation was present in both treated and untreated piglets.

**(ii)** In the clinical study, deep gray matter Lac/NAA was the most accurate predictor of long term adverse neurological outcome following NE; importantly the predictive accuracy of Lac/NAA was unaltered by preceding therapeutic hypothermia.

**Conclusions:** **(i)** Early thalamic Lac/NAA predicted the subsequent trajectory of energy disruption; this has importance in understanding the relevance of very early MRS studies in babies. White matter lactate/Cr at 40-48h correlated best with quantitative immunohistochemistry (especially TUNEL positive cells) and this relationship was present with and without preceding therapeutic intervention. **(ii)** In babies with neonatal encephalopathy, the predictive accuracy of Lac/NAA was unaltered by therapeutic hypothermia.

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## **Chapter 1 - Introduction**

# 1.1 Epidemiology

## General introduction

Cerebral palsy (CP) remains the largest single cause of childhood disability in the developed world with a prevalence of 2 per 1000 live births (Surman, Bonellie et al. 2006). Historically, the first clinical description of spastic diplegia was by William John Little, an orthopaedic surgeon who specialized in childhood deformities. He first presented a series of lectures titled *Deformities of the Human Frame* (Little 1843) and later presented a paper linking difficult labour, prematurity and neurological problems later in life (Little 1862). Since then, decades of debate have ensued on the exact relationship between hypoxia-ischemia (HI) and brain injury. It was not until the early 1950s that measures were undertaken to establish firm epidemiologic and physiologic relationships between neurologic abnormalities of childhood and the events of labour. New insights from research into the etiology of cerebral palsy have recently transformed the old concept that most cases of cerebral palsy begin during labour.

## Definitions

The terms “asphyxia”, “hypoxia”, “hypoxemia” and “hypoxia-ischemia” have often been used interchangeably. It is essential to understand the exact definition of these words to establish the relationship with neurological outcome in later childhood. Tissue hypoxia is the central process, and it is defined as diminished oxygen delivery to the tissues. It is intracellular hypoxia, which initiates anaerobic metabolism, metabolic acidosis and results in cell death. The neonatal brain is very sensitive to the effects of tissue hypoxia. Tissue hypoxia can be produced either by hypoxemia, which is defined as decreased oxygen concentrations within the blood and/or ischemia, which is defined as decreased blood flow to the tissues. Asphyxia refers to impairment in blood gas exchange resulting in hypoxemia, hypercapnia and acidosis.

This definition of asphyxia was by a task force for the world federation of neurology group (Bax and Nelson 1993).

In addition, other terms including neonatal encephalopathy (NE) and hypoxic-ischemic encephalopathy (HIE) also need clear definition as often they have been used incorrectly. Neonatal encephalopathy is defined as a clinical syndrome of disordered neurological function in a term or near term infant in early neonatal period and is usually manifested by difficulties in initiating and maintaining respiration, depressed tone and reflexes, altered consciousness levels and commonly associated with seizures (Nelson and Leviton 1991). Neonatal encephalopathy may be associated with variety of causes including genetic, metabolic, infective conditions and others (Hankins and Speer 2003). However, HIE is one form of NE where there is strong evidence of intrapartum asphyxia resulting in neurologic depression with or without seizures. In the past, it was largely assumed that most cases of NE were due to adverse intrapartum events. However, the term NE is simply a clinical description of disturbed neurological function regardless of its etiology or pathogenesis. So the term HIE refers to the sub-set of cases of NE where there is evidence of a recent, or intrapartum hypoxia-ischemia as a cause. Hypoxic ischemic encephalopathy is associated with multiple factors including maternal, cord, placental and neonatal factors. It could be due to acute or chronic event.

### ***Incidence***

The incidence of NE reported from various studies ranges from 2 to 6 per 1000 live births (Brown, Purvis et al. 1974, Badawi, Kurinczuk et al. 1998, Ellis, Manandhar et al. 2000, Evans, Rigby et al. 2001). The incidence of HIE varies from 1 to 8 per 1000 live births from various studies (Kurinczuk, White-Koning et al. 2010)

There have been some difficulties in estimating incidence accurately from these studies. One of them is the use of several case definitions in the studies. In addition,

some of them are hospital based and some are population-based studies. This is a problem because hospital based studies have higher incidence than population based studies as they are conducted in tertiary referral centres. The other issue is the time period of the study as most of the hospital-based studies are older than population based studies. There is a decrease in the incidence from 1970s through to 1990s (Hull and Dodd 1992, Smith, Wells et al. 2000).

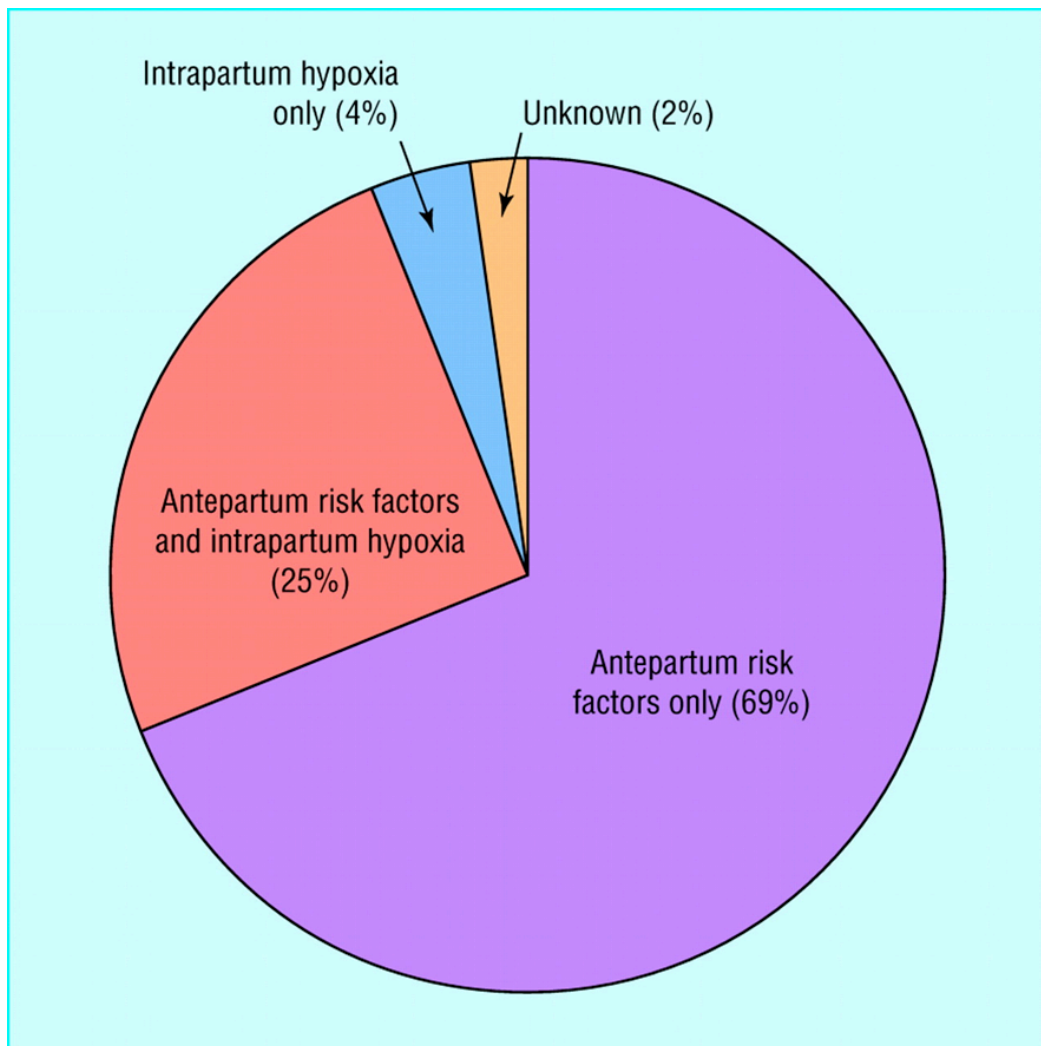
Furthermore, the same denominator was not used in all studies, as gestational age was different in different studies. In addition to methodological differences, the basic data, which is from various part of the world, may not be applicable to the local population to estimate incidence. In a review of the relationship between NE and cerebral palsy in 2003, the American College of Obstetricians and Gynaecologists and the Academy of Pediatrics concluded that the best estimate of the incidence of NE comes from population data and was 1.9 to 3.8 per 1000 (American College of Obstetricians and Gynecologists 2002). Finally, based on population studies with similar inclusion and denominator criteria, Kurinczuk et al estimated the incidence of NE to be 3.0 per 1000 live births (95%confidence interval 2.7-3.3), incidence of HIE to be 1.5 per 1000 live births (95% confidence interval 1.3-1.7) (Kurinczuk, White-Koning et al. 2010)

### ***Risk factors***

Risk factors for NE are diverse and identifying them helps us understand the pathogenesis and develop preventive strategies. There was a difficulty in identifying the risk factors, as there was no universally accepted case definition and no properly designed studies. Various intrapartum and antepartum risk factors are present in NE as demonstrated in the case control study from Western Australia (Badawi, Kurinczuk et al. 1998, Badawi, Kurinczuk et al. 1998). Intrapartum risk factors included maternal pyrexia, persistent occipito-posterior position and acute intrapartum events. In this study, around 30% of cases of NE are associated with the



evidence of intrapartum hypoxia (Figure 1.1) and the rest of the cases are due to other risk factors. This is very different to the case control study from Nepal, where around 60% of NE cases had evidence of intrapartum hypoxia (Ellis, Manandhar et al. 2000). Intrapartum risk factors from this study included non-cephalic presentation, prolonged rupture of membranes, cord prolapse and uterine rupture.



**Figure 1.1:** Distribution of risk factors for neonatal encephalopathy. The Western Australia case control study (Badawi, Kurinczuk et al. 1998) Permission to reproduce this figure has been granted by BMJ Publishing Group Ltd.

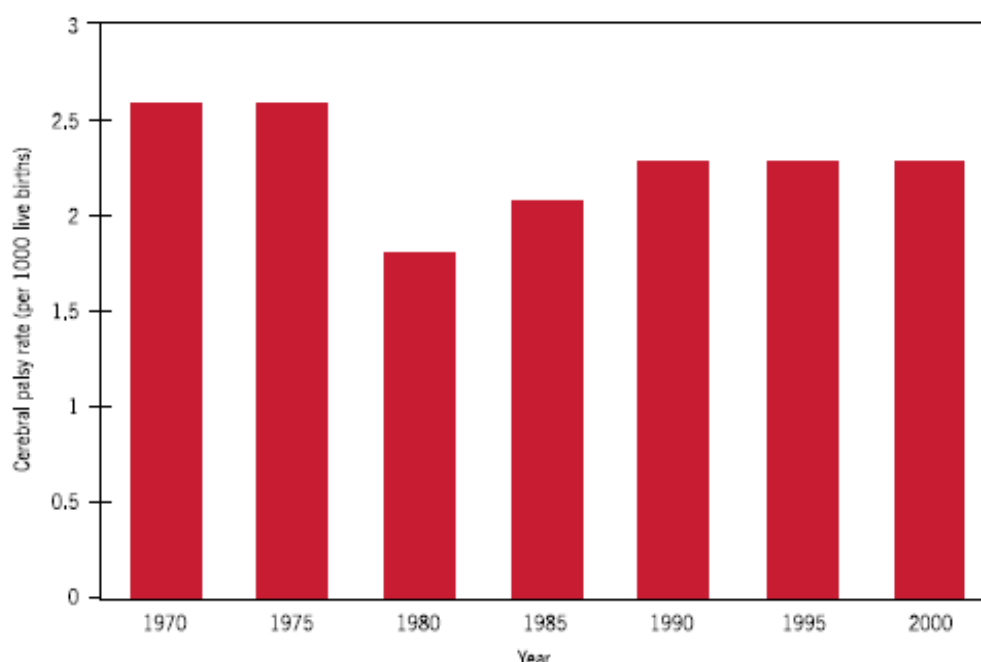
### **The role of NE and HIE in causation of cerebral Palsy**

Cerebral palsy (CP) is a group of disorders of the central nervous system manifested by aberrant control of movement or posture, present since early in life and not the result of recognized progressive disease. The Surveillance of Cerebral Palsy in

Europe (SCPE) agreed a similar definition (SCPE 2000). The key elements in this definition are: 1) Cerebral palsy is a group of disorders which are permanent but not unchanging; 2) The condition involves a disorder of movement and/or posture and of motor function; 3) The condition is due to a non-progressive interference, lesion or abnormality of the developing immature brain. It remains a common and disabling condition in which the diagnosis may not be confirmed until 5 years of age. It is well known for decades that children born preterm and low birth weight have increased risk of CP (Fawke 2007, Platt, Cans et al. 2007).

Decades ago, when electronic fetal monitoring was first introduced, it was thought that intrapartum hypoxia-ischemia accounted for 50% of perinatal mortality and morbidity (Quilligan and Paul 1975). Subsequently, population based studies have demonstrated that intrapartum hypoxia-ischemia was present in small percentage of term infants who developed CP ranging from 8% in Australia and 28% in Sweden (Blair and Stanley 1988, Hagberg, Hagberg et al. 2001). The rate of CP has not decreased in the past 30 years (Figure 1.2) despite the fall in the incidence of HIE, widespread use of electronic fetal monitoring and increase in caesarean section (Hull and Dodd 1992, Clark and Hankins 2003). Findings from a hospital based study from 2 tertiary neonatal units, which included 351 term infants with neonatal encephalopathy presenting within 72 hours of age, demonstrated that events in the immediate perinatal period are most important in neonatal brain injury (Cowan, Rutherford et al. 2003). They defined intrapartum asphyxia as the presence of at least 3 of the following: (1) late decelerations on fetal monitoring or meconium staining, (2) delayed onset of respiration, (3) arterial cord blood pH < 7.1, (4) Apgar score < 7 at 5 minutes, and (5) multi-organ damage. On the basis of magnetic resonance imaging (MRI) within 2 weeks of birth or postmortem examination, 80% of the neonates with NE and asphyxia had lesions of the deep gray matter, cortex, or white matter consistent with an evolving hypoxic-ischemic insult and 69% of

neonates with only seizures within 3 days of birth had acute ischemic or hemorrhagic lesions (Cowan, Rutherford et al. 2003).



**Figure 1. 2: Cerebral Palsy rates per 1000 live births 1970 – 2000 (Clark and Hankins 2003)** *Permission to reproduce this figure has been granted by Elsevier.*

Historically, the factors used to define perinatal asphyxia, such as meconium-stained amniotic fluid and Apgar scores, were not specific to the disease process leading to neurologic damage. When 110 term infants with severe metabolic acidosis were examined, it was concluded that even severe acidosis in umbilical cord blood is insufficient evidence of intrapartum hypoxia-ischemia profound enough to cause neurologic damage (Fee, Malee et al. 1990). From a population based study in Western Australia, there was no evidence of intrapartum hypoxia in over 70% of cases of newborn encephalopathy and that isolated pure intrapartum hypoxia accounted for only 4% of moderate to severe newborn encephalopathy (Figure 1.1). They further observed that intrapartum hypoxia might have been superimposed on preconceptional or antepartum risk factors with pre-existing insult in 25% of cases. In 1988, Blair and Stanley reported similar results; in only 8% of all of the children with

spastic cerebral palsy was intrapartum asphyxia the possible cause of their brain damage (Blair and Stanley 1988). It can also be stated with certainty that the pathway from an intrapartum hypoxic–ischemic injury to subsequent cerebral palsy must progress through NE and that HIE is but a minor component of the broader diagnostic category of neonatal encephalopathy.

Since early 1990s, considerable efforts have been put into define the criteria to diagnose intrapartum hypoxia sufficient to cause CP. It was first proposed by The American College of Obstetricians and Gynecologists (ACOG) and the criteria were further refined by the International Cerebral Palsy Task Force Consensus Statement (MacLennan 1999). Again the criteria have again been reviewed and knowledge updated by the ACOG and American Academy of Pediatrics Task Force on Neonatal Encephalopathy and Cerebral Palsy (American College of Obstetricians and Gynecologists 2002). These statements emphasise the use of multiple criteria for the diagnosis (Table 1.1). The recent Swedish study suggests that an increasing number of term infants fulfil now the ACOG criteria of intrapartum-events severe enough to cause CP(Himmelman, Hagberg et al. 2010). A recent systematic review on the association between intrapartum hypoxia-ischemia and NE at term reported the proportion of cerebral palsy associated with intrapartum hypoxia-ischemia was only 14.5% (Graham, Ruis et al. 2008). From the same study, the incidence of an umbilical arterial pH <7.0 at birth was 3.7 per 1000 births and the incidence of HIE was 2.5 of 1000 live births.

<i>Essential criteria (must meet all four)</i>
1. Evidence of a metabolic acidosis in intrapartum fetal, umbilical arterial cord, or very early neonatal blood samples (pH <7.00 and base deficit ≥12 mmol/l)
2. Early onset of severe or moderate neonatal encephalopathy in infants of ≥34 weeks' gestation
3. Cerebral palsy of the spastic quadriplegic or dyskinetic type
4. Exclusion of other identifiable aetiologies such as trauma, coagulation disorders, infectious conditions, or genetic disorders
<i>Criteria that together suggest an intrapartum timing but by themselves are non-specific:</i>
5. A sentinel (signal) hypoxic event occurring immediately before or during labour
6. Sudden and sustained fetal bradycardia or the absence of fetal heart rate variability in the presence of persistent, late, or variable decelerations usually after a hypoxic sentinel event when the pattern was previously normal
7. Apgar scores of 0–3 beyond 5 min
8. Onset of multisystem involvement within 72 h of birth
9. Early imaging evidence of acute non-focal cerebral abnormality

**Table 1.1:** Criteria to define an acute intrapartum event according to the ACOG and International Consensus criteria (American College of Obstetricians and Gynecologists 2002) Permission to reproduce this table has been granted by Wolters Kluwer Health.

The Apgar score was first proposed more than 50 years ago and has become a standard method of assessing the condition of a newborn infant at birth (Apgar 1953). This assessment entails the scoring of 0, 1 and 2 on the five physiological characteristics: pulse rate, respiratory effort, colour, muscle tone and response to stimulation. A retrospective study of Apgar scoring in children with CP demonstrated that the predictive value of the score for CP is very low as 73% of children with CP had high score at 5 minutes of age and 83% of infants with low score at 10 minutes of age were free of major disability at early school age (Nelson and Ellenberg 1981). Assessment of the newborn within minutes of delivery, whilst a useful measure to alert the clinician to potential evidence of an intrapartum event, is not able to give prognostic information.

The clinical picture of neonatal encephalopathy is graded according to Sarnat and Sarnat (Table 1.2) (Sarnat and Sarnat 1976). The risk of death or major disability in infants diagnosed with mild encephalopathy was 2%, moderate encephalopathy was

19% and severe encephalopathy was 63%(MP Collins 2002).

	Mild	Moderate	Severe
Level of Consciousness	Hyperalert	Lethargic	Stuporose
Neuromuscular Control			
Muscle tone	Normal	Mild Hypotonic	Flaccid
Posture	Normal	Flexion	Decerebrate
Tendon reflex	Hyper-reflexia	Hyper-reflexia	Absent
Clonus	Present	Present	Absent
Pupils	Dilated	Constricted	Poorly reactive
Seizures	None	Common	Frequent/intractable
Moro Reflex	Strong	Weak/incomplete	Absent
Suck Reflex	Normal/weak	Weak/absent	Absent

**Table 1.2:** Grading of neonatal encephalopathy by clinical evaluation - modified(Sarnat and Sarnat 1976)

Amplitude-integrated electroencephalogram (aEEG) monitoring with the cerebral function monitor (CFM) was first described almost 40 years ago (Maynard, Prior et al. 1969). The aEEG allows continuous long-term monitoring of background activity and has been used in the care of neonates with encephalopathy as it has allowed early prediction of neurodevelopmental outcome, especially when used in conjunction with clinical neurological evaluation. In a recent meta-analysis, which included 8 studies, there was an overall sensitivity of 91% (95% CI 87-95) and a negative likelihood ratio of 0.09 (95% CI .06-.15) for aEEG tracings to accurately predict poor outcome in infants with HIE (Spitzmiller, Phillips et al. 2007).

There were more findings in the follow up, population based study of term infants

with NE in Western Australia (Badawi, Felix et al. 2005). Nearly one out of every four term children with CP has a history of moderate or severe neonatal encephalopathy. Cerebral palsy in this context is more likely to affect males, to be severe, and to be accompanied by epilepsy, lack of speech, and severe cognitive impairment. Moreover, of those who go on to develop CP following neonatal encephalopathy, about one out of every five will die in the first 5 years of life. Infants born in developing countries have a high risk of intrapartum-related injury (Lawn, Kinney et al. 2009). Almost one quarter of the world's 4 million annual neonatal deaths are caused by perinatal asphyxia and 99% of these deaths occur in low and mid-resource settings (Lawn, Cousens et al. 2005).

### **Summary**

The relationship between intrapartum hypoxia-ischemia, neonatal encephalopathy and cerebral palsy is more complex than that originally described by Little 150 years ago. The causal chain of events resulting in NE is complex, multifactorial and far from completely understood. Hypoxic–ischemic encephalopathy represents a small subset of neonatal encephalopathy and an even smaller contributor to cerebral palsy. Several promising lines of enquiry and hypotheses relating to potentially modifiable risk factors were generated by previous epidemiological studies although these studies are now well over a decade old and there have been substantial changes in clinical practice and clinical governance since their publication. Advances in magnetic resonance (MR) brain imaging have led to a much better understanding of the relationship between brain injury and the likely timing of the causal insult in the antepartum and peripartum periods (Cowan, Rutherford et al. 2003). Amplitude integrated EEG is becoming routine in clinical practice. All of these developments merit further investigation in appropriately sized population-based case–control studies.

## **1.2 Mechanisms of brain injury after hypoxia-ischemia**

### **Introduction**

Hypoxic-ischemic brain injury is a predominant form of all brain injury encountered in the perinatal period and it is a major cause of subsequent neurological disability in term infants. Research has provided much greater insights into the pathogenesis of hypoxic-ischemic brain injury in the foetus and neonate, over the last two decades through both clinical and animal experiments. Understanding these mechanisms help the clinician understand the potential for recovery and to work on potential targets for intervention.

### **The Neurotoxic Cascade**

#### **Excitotoxicity**

The principle process responsible for hypoxic-ischemic damage to neurons is excitotoxicity (Choi and Rothman 1990). Excitotoxicity refers to cell death mediated by excessive stimulation of extracellular excitatory amino acid receptors (Choi and Rothman 1990). Glutamate is a neuro-transmitter, present in excitatory presynaptic terminals throughout the brain. There are three types of glutamate receptors: N-methyl-D-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methylisoazole-4- propionic acid (AMPA), and kainic acid (KA). Normally these receptors mediate physiologic excitatory effects of the glutamate. Under normal conditions, neuronal and glial re-uptake pumps remove glutamate rapidly from the extracellular space before toxicity occurs (Schousboe 1981).

In hypoxia-ischemia, early severe disruption of function in glutamate synapses was observed and the activity of these reuptake pumps was markedly reduced



(Silverstein, Naik et al. 1991, Tao, Lu et al. 2001). Abnormally raised levels of glutamate have been demonstrated in the brain following asphyxia in animal models and in the cerebrospinal fluid (CSF) of neonates following severe hypoxic-ischemic insult (Hagberg, Andersson et al. 1987, Johnston, Nakajima et al. 2002). A study using  $^1\text{H}$ -MRS (proton magnetic resonance spectroscopy) also demonstrated that glutamate levels were elevated in the brains of infants with severe hypoxic-ischemic encephalopathy (Pu, Li et al. 2000). The energy for these reuptake pumps is derived from the anaerobic metabolism of glucose (Magistretti, Pellerin et al. 1999). Severe hypoglycaemic episodes have been shown to result in increased levels of extracellular glutamate (Ichord, Northington et al. 1999).

Further evidence for the involvement of excitatory neurotransmitters in perinatal brain injury comes from the observation that areas of the term neonatal brain susceptible to damage which include the putamen, thalamus and the peri-rolandic cerebral cortex, have the highest density of glutamate utilising synapses (Johnston 1995, Sie, van der Knaap et al. 2000). Also, neuroprotection seen with the use of glutamate receptor blockers when administered after termination of the insult supports the central role of glutamate in brain injury (McDonald, Silverstein et al. 1990). The immature developing brain is far more susceptible to excitotoxicity than mature adult brain because of the sub unit compositions of the NMDA receptors expressed during development (Monyer and Seeburg 1993, Holmes and Ben-Ari 2001)

### **Calcium influx**

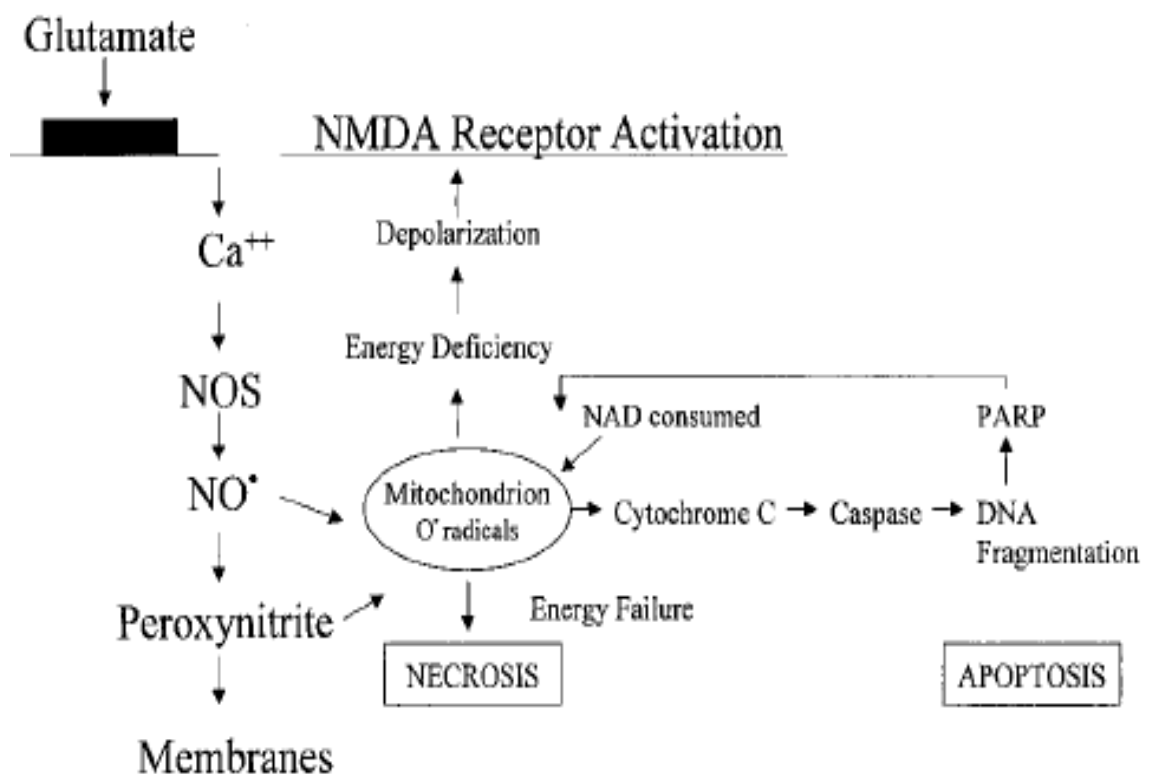
Membrane depolarization, along with high levels of synaptic glutamate, produces maximal opening of the channels and flooding of calcium and sodium into neurons (Choi and Rothman 1990). Normally mitochondria buffer intracellular calcium. However, the calcium 'overload' causes mitochondrial dysfunction (Stout, Raphael et

al. 1998, Johnston, Nakajima et al. 2002). Calcium influx initiates multiple pathways(Cheung, Bonventre et al. 1986). It activates many different enzymes including lipases and proteases. Lipase activation results in breakdown of cell membranes and production of prostaglandins and leukotriens. Activation of proteases causes activation of the caspase cascade leading to cellular apoptosis. Oxygen free radicals are generated as well during this process through activation of nitric oxide synthase (NOS) and xanthine oxidase by excessive calcium(Volpe 2001). It also uncouples oxidative phosphorylation, resulting in energy (ATP) depletion.

### **Free radical injury**

Reactive oxygen and nitrogen species are produced during hypoxia-ischemia and reperfusion following hypoxia-ischemia (Bagenholm, Nilsson et al. 1998). These species are highly reactive compounds which can generate more free radicals through interaction with cellular components. This causes peroxidation of fatty acids, which results in cell membrane injury and necrosis. Free radical also causes cell death by apoptosis through activation of the apoptotic gene(Blomgren and Hagberg 2006). Following hypoxia-ischemia, the inhibition of neuronal nitric oxide synthase improved long-term outcome in neonatal rats (van den Tweel, Peeters-Scholte et al. 2002). Furthermore, it has been demonstrated that neonatal mice lacking neuronal nitric oxide synthase have been shown to be less susceptible to hypoxic-ischemic injury(Ferriero, Holtzman et al. 1996). Also, melatonin, a neurohormone with potent anti-oxidant activity, has been shown to have neuroprotective action on rat brains following hypoxia-ischemia and reperfusion(Wakatsuki, Okatani et al. 2001). In addition, melatonin was also neuroprotective following excitotoxic brain injury in newborn mice (Husson, Mesples et al. 2002).

In summary, following hypoxia-ischemia, a cascade of neurotoxic events was initiated that centres on the excitotoxicity by glutamate which, along with a reduced membrane potential, act to open the NMDA receptor. This results in influx of calcium into the cells. This leads to further mitochondrial dysfunction and production of free radical species which results in cell death through apoptosis and necrosis (Figure 1.3).



**Figure1.3:** Summary of neurotoxic cascade (Johnston, Trescher et al. 2001) Hypoxic ischemia triggers opening of NMDA-type glutamate receptor-operated channels in the cytoplasmic membrane, allowing Ca to flood into the cytoplasm. Ca fluxed through NMDA channels can activate Ca sensitive enzymes such as nitric oxide synthase (NOS), producing the free radical gas nitric oxide (NO), which is toxic alone or when combined with superoxide ions to form even more toxic peroxynitrite. One target of NO and peroxynitrite is mitochondria, which generate their own supply of oxygen free radicals under hypoxic conditions. cytochrome c protein released from distressed mitochondria triggers the activation of cysteine-dependent aspartate-directed proteases (caspases) such as caspase 3 that lead to fragmentation of DNA and many other actions. DNA fragmentation in turn can trigger activation of poly (ADP-ribose) polymerase (PARP), a nuclear enzyme that facilitates DNA repair. Oxidative failure from hypoxic ischemia combined with a reduction in NAD can further impair mitochondrial function and reduce energy needed to maintain membrane potentials. A fall in membrane potential leads to passive opening of NMDA channels, worsening and extending the excitotoxic cascade. Permission to reproduce this figure has been granted by Nature Publishing Group.

## **The role of inflammation**

### **Role of Cytokines**

One of the mechanisms of brain injury, following cerebral hypoxia-ischemia, is through activation of cytokines. Pro inflammatory cytokines are released in response to hypoxia-ischemia (Hagberg, Gilland et al. 1996, Savman, Blennow et al. 1998). Both interleukin1 beta (IL-1  $\beta$ ) and tumour necrosis factor-alpha (TNF $\alpha$ ) have been shown to increase in the brain 4-6 h after the insult. High levels of IL1, 6, 13 and TNF $\alpha$ , were seen in infants who later developed cerebral palsy(Nelson, Dambrosia et al. 1998). These cytokines including TNF $\alpha$ , IL-1 $\beta$  and interferon gamma (INF $\gamma$ ) have toxic effects on vulnerable oligodendrocyte precursors and on neurons resulting in brain injury(Volpe 2001). Further evidence for the role of these cytokines comes from experimental animal models. Treatment with interleukin receptor antagonist ameliorates brain injury following hypoxia-ischemia(Hagan, Barks et al. 1996, Hagberg, Gilland et al. 1996). In addition, it has been shown that deletion of IL- 18 and IL-1 $\beta$  reduced brain injury following hypoxia-ischemia(Hedtjarn, Mallard et al. 2005). Another group of cytokines (anti-inflammatory), including transforming growth factor beta1 (TGF- $\beta$ 1) and interleukin-10 (IL-10) are released following cerebral ischemia (Raivich, Jones et al. 1999). TGF- $\beta$ 1 inhibits the activation of brain microglia (Suzumura, Sawada et al. 1993), and inhibits the microglial production of neurotoxic molecules such as nitric oxide, superoxide and IL-1 (Vincent, Tilders et al. 1997). In adult, application of TGF- $\beta$ 1 strongly reduces the infarct size in cerebral ischemia (Vincent et al 1997). Systemic administration of IL – 10 reduced the excitotoxic brain injury in newborn mice (Mesples, Plaisant et al. 2003). So it is critical in maintaining the balance between pro and anti-inflammatory cytokines for neuroprotection.

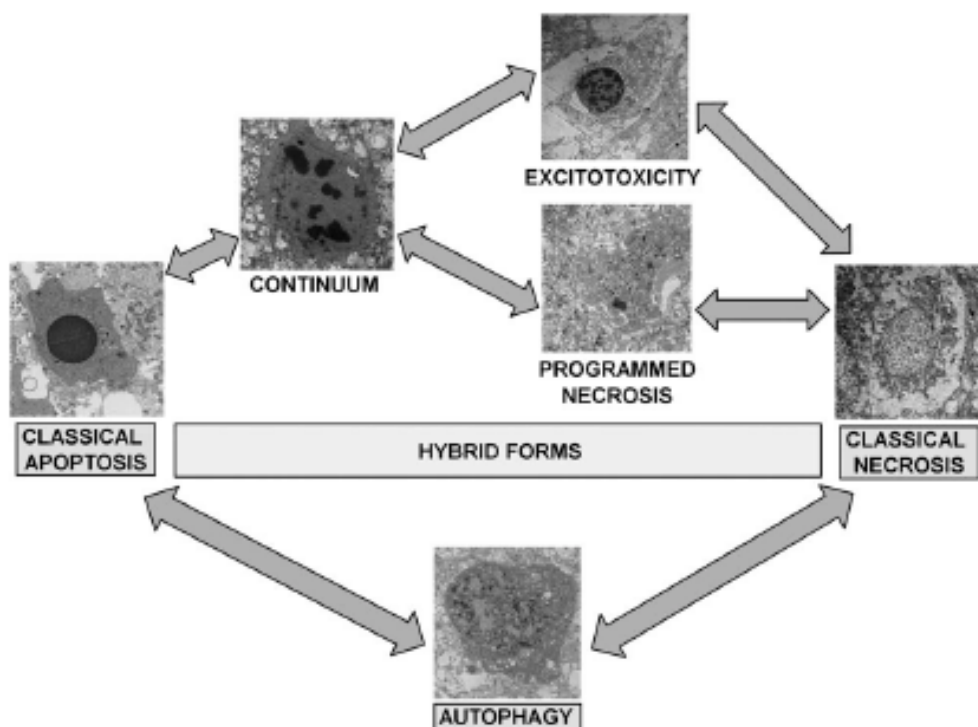
## **Role of Microglia**

Microglia are a sub-type of monocytes. They have specific roles in immune surveillance, cytokine production, antigen presentation, complement activation and phagocytosis within the developing central nervous system. Microglia are highly ramified cells in the resting state with a tertiary and quaternary branching structure which is integral to their role in immune surveillance (Raivich 2005). In addition to the resting state, microglia can take on a deramified, amoeboid phagocytic phenotype when needed. Microglia are activated along with neutrophil infiltration, during cerebral hypoxia-ischemia and reperfusion (Hudome, Palmer et al. 1997). Activated microglia were observed from 4 hours after reperfusion and continued to increase over the next 48 hours (Bona, Andersson et al. 1999). Microglial cells release neurotoxic substances such as glutamate, cytokines, reactive oxygen and nitrogen species. Activation of microglia has been shown in animal models of excitotoxic and hypoxic-ischemic brain injury (Hagberg, Peebles et al. 2002). Minocycline, a second-generation tetracycline antibiotic reduces microglial activation (Zemke and Majid 2004) and prevents brain injury when administered prior to, or after hypoxia-ischemia (Arvin, Han et al. 2002).

## 1.3 Neuronal cell death in cerebral hypoxia-ischemia

### Introduction

Cell death is a fundamental biological process that is relevant to normal histogenesis and also to the pathogenesis of tissue damage. It has physiological and pathological importance. Cell death was first classified into apoptotic or necrotic in 1970s, based on the appearances under the microscope. Necrosis is a rapidly occurring form of cell death that is common in the acute phases of severe cerebral insults and has been attributed, in part, to alterations in ionic homeostasis. In contrast, apoptosis is a delayed form of cell death that occurs as the result of activation of genetic and molecular mechanisms.



**Figure 1. 4:** Cell death phenotypes in experimental neonatal HI brain injury (Northington, Chavez-Valdez et al. 2011) Various relationships between the classical and hybrid forms of cell death are suggested by their appearance on electron microscopy as represented by the arrows in the figure. Autophagocytic-appearing neurons with large numbers of cytoplasmic vacuoles, partially condensed nuclear chromatin, and preservation of cellular integrity are also found after neonatal HI. Autophagocytic cell death and apoptosis and possibly necrosis also exist on a continuum. Permission to reproduce this figure has been granted by John Wiley and Sons.

With new insights from the research into the mechanisms underlying apoptosis, it is being recognized that both apoptosis and necrosis are likely part of continuum of cell death (Figure 1.4) with similar operative mechanisms rather than two different entities (Northington, Chavez-Valdez et al. 2011).

### **Characteristics of necrosis**

Necrosis is a lytic destruction of individual or groups of cells. Necrosis is the major cell death in brain following acute neonatal HI injury in most animal models (Martin, Brambrink et al. 2000, Northington, Ferriero et al. 2001). Classical necrosis is defined by cytoplasmic swelling, nuclear dissolution (karyolysis), and is thought to be caused by rapid and severe failure to sustain cellular homeostasis. The process of necrosis involves damage to the structural and functional integrity of the cell plasma membrane and associated enzymes, abrupt influx and overload of ions, and rapid mitochondrial damage and energetic collapse (Martin, Brambrink et al. 2000, Golden, Brambrink et al. 2001). Subsequently, there is dissolution of organelles, rupture of the plasma membrane with leakage of cellular contents into the extracellular space, and random DNA degradation following histone proteolysis. Metabolic inhibition and oxidative stress from reactive oxygen species (ROS) are major culprits in triggering necrosis. Necrosis usually affects large groups of adjacent cells and is associated with inflammatory reaction. Recently, it has been shown that cell necrosis might involve the activation of specific signalling pathways or programs to result in cell death (Festjens, Vanden Berghe et al. 2006).

### **Characteristics of apoptosis**

Apoptosis is an orderly process of dismantling of single cell or group of cells into components. Apoptosis as a mode of cell death following moderate hypoxic injury

has been shown in experimental models (Beilharz, Williams et al. 1995). It is one example of programmed cell death (PCD) that is ATP-driven and sometimes a gene transcription-requiring and caspase-dependent process (Tata 1966). In most classic descriptions of apoptosis, the earliest changes occur within the nucleus. Cells undergoing apoptosis exhibit shrinkage of the cytoplasm and condensation of nuclear material into "clumps" (Walker, Harmon et al. 1988). Then, the nucleus undergoes fragmentation and the endoplasmic reticulum fuses with the plasma membrane forming vesicles and convoluting the surface of the plasma membrane. Finally, there is cellular fragmentation forming membrane-bound apoptotic bodies that contain intact cytoplasmic organelles and nuclear fragments, and these apoptotic bodies are subsequently phagocytosed by healthy neighbouring cells. The biochemical hallmark of apoptosis is the internucleosomal cleavage of DNA into double-stranded fragments, mediated by calcium and magnesium dependent endonuclease (Wyllie 1980). There are key differences between apoptosis and necrosis morphologically (Table 1.3). First, apoptotic cells exhibit loss of cytoplasm while necrotic cells have cytoplasmic swelling. Second, nuclear changes precede plasma membrane changes in apoptosis while the reverse is observed during necrosis. Third, apoptotic cells maintain plasma membrane integrity while necrotic cells undergo plasma membrane rupture.



Component	Apoptosis	Necrosis	Continuum
Cytosol	Cytoplasmic condensation and shrinkage, and plasma membrane blebbing; generally intact-appearing mitochondria, though mitochondria may appear edematous	Complete organelle disruption	Variably swollen or ruptured organelles within a darkened/partially condensed cytoplasm with vacuolar changes in the perikaryon and occasional autophagocytic inclusions
Cell membrane	Cytoplasmic membrane fully intact	Swelling and rupture of the cell membrane	Intact cell membranes
Nucleus	Large spherical/globular or crescent-shaped chromatin clumps tightly compacted into a few (<3-4) fragments whose appearance is reminiscent of developmental programmed cell death; transformation of the nucleolus into separate granular masses, which unwind; preservation of nuclear membrane	Watery, "clock face" – appearing nucleus with widely dispersed and very small clumps of chromatin within an intact nuclear membrane	Condensed, but not tightly compacted, chromatin clumps less regular in shape and divided into more fragments (>3) than that seen in apoptosis, with partially intact nuclear membrane

**Table 1.3: Characteristics of apoptosis and necrosis (Northington, Zelaya et al. 2007)**  
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## **DNA fragmentation in apoptosis and necrosis**

Various biochemical markers have been used to classify cell death. The most extensively used marker for cell death is based on the integrity of nuclear DNA (Wyllie 1980). Genomic DNA degradation indicates an irreversible stage of cell death. In necrotic cells, DNA undergoes random fragmentation following histone proteolysis. However, histones remain intact in apoptotic cells, so DNA is cleaved into multimers of 180 to 240 base pairs (Wyllie 1980). Genomic DNA from necrotic cells exhibits a single vertical band on electrophoresis ("smear pattern"). But in apoptosis, genomic DNA will exhibit a "ladder pattern" on agarose gel electrophoresis (Wyllie 1980). However, biochemical markers of cell death vary from one cell type to another. In some cell cultures, apoptotic cell death occurs in the absence of demonstrable internucleosomal DNA fragmentation. The development of a technique to detect DNA fragmentation at the cellular level provided a new approach to diagnose cell death. This strategy is based on end-labelling single-strand or double-strand DNA breaks using terminal deoxynucleotidyl transferase. This approach is called the terminal transferase-mediated dUTP nick-end labelling (TUNEL) method (Gavrieli, Sherman et al. 1992). This method is quite sensitive for detection of cells with DNA fragmentation, however, this does not discriminate among apoptotic and necrotic cell death (Grasl-Kraupp, Ruttkay-Nedecky et al. 1995).

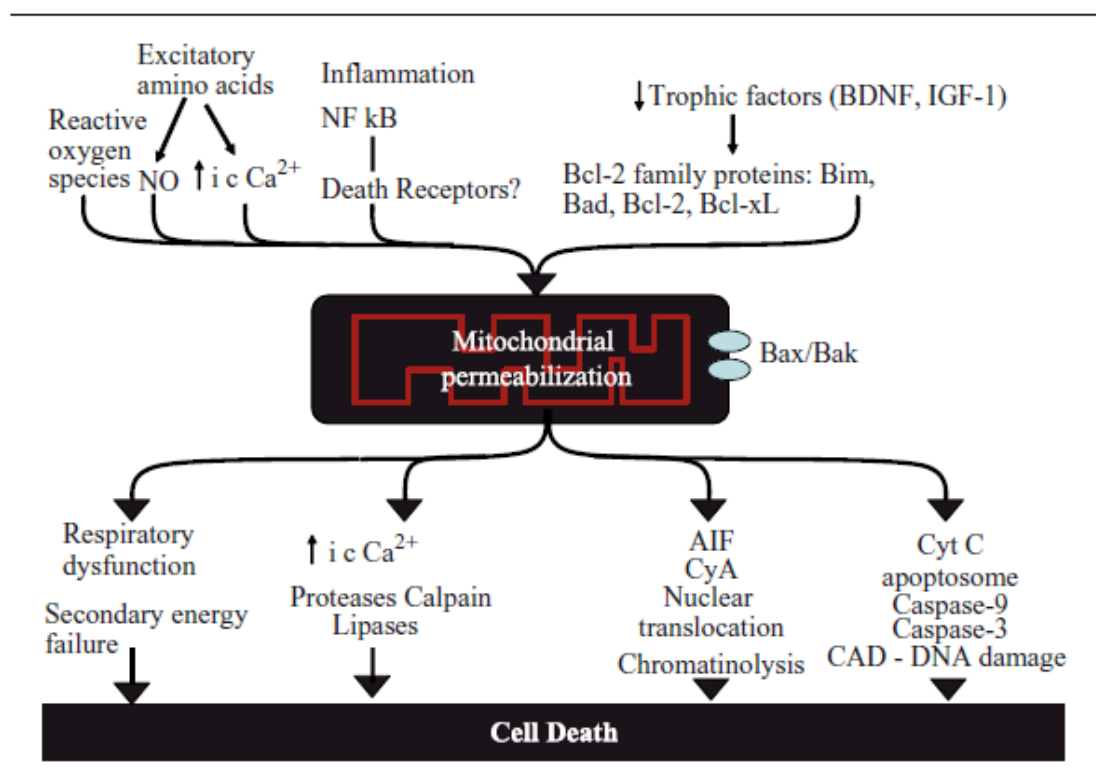
## **Other forms of cell death**

Apoptosis is only one form of PCD. There are other forms: Caspase-independent apoptosis and non-apoptotic PCD (Lockshin and Zakeri 2002, Festjens, Vanden Berghe et al. 2006). Variants of classical apoptosis and non-classical apoptosis occur in brain during normal development and during pathogenesis, particularly following hypoxia-ischemia (Northington, Ferriero et al. 2001, Northington, Ferriero et al.

2001). Excitotoxins can induce non-classical form of apoptosis (Portera-Cailliau, Price et al. 1997). Autophagy is a highly regulated process that allows a cell to recycle its own cytoplasm and organelles. The degradation of organelles and long-lived proteins is carried out by the lysosomal system. Autophagy is termed as type 2 PCD. Presence of lysosomal vacuoles is the hallmark of autophagy. It is induced under starvation, differentiation, and normal growth control to maintain homeostasis and survival (Shintani and Klionsky 2004). Autophagy may have a significant role in pathogenesis of brain injury following HI (Ginet, Puyal et al. 2009). Inhibition of autophagy with 3-methyladenine up to 4 hours after focal ischemia is neuroprotective in p12 rats (Puyal and Clarke 2009). Conversely, induction of autophagy immediately following neonatal global HI in mice may be an endogenous neuroprotective mechanism (Carlioni, Buonocore et al. 2008).

### **Mechanisms underlying cell death**

Multiple apoptotic pathways have been shown to be involved in neonatal hypoxic-ischemic cell death. Considerable data have accrued indicating that mitochondria participate in the critical effector stage of apoptosis. The mechanisms leading to secondary brain injury include excitatory amino acids, intracellular calcium, nitric oxide/reactive oxygen species, immuno-inflammatory activation, trophic factor withdrawal, and Bcl-2 family proteins. These perpetrators converge on mitochondria, and at a certain level of stress mitochondrial outer membrane permeabilization occurs, which results in irreversible cell death (Figure 1.5).



**Figure 1. 5:** Mitochondrial outer membrane permeabilization AIF, apoptosis-inducing factor; CAD, caspase-activated Dnase; cyA, cyclophilin A; cytC, cytochrome C; BDNF, brain-derived neurotrophic factor; ic, intracellular; IGF-1, insulin-like growth factor 1; NF-κB, nuclear factor kappa B. (Hagberg, Mallard et al. 2009). Permission to reproduce this figure has been granted by Sage publications.

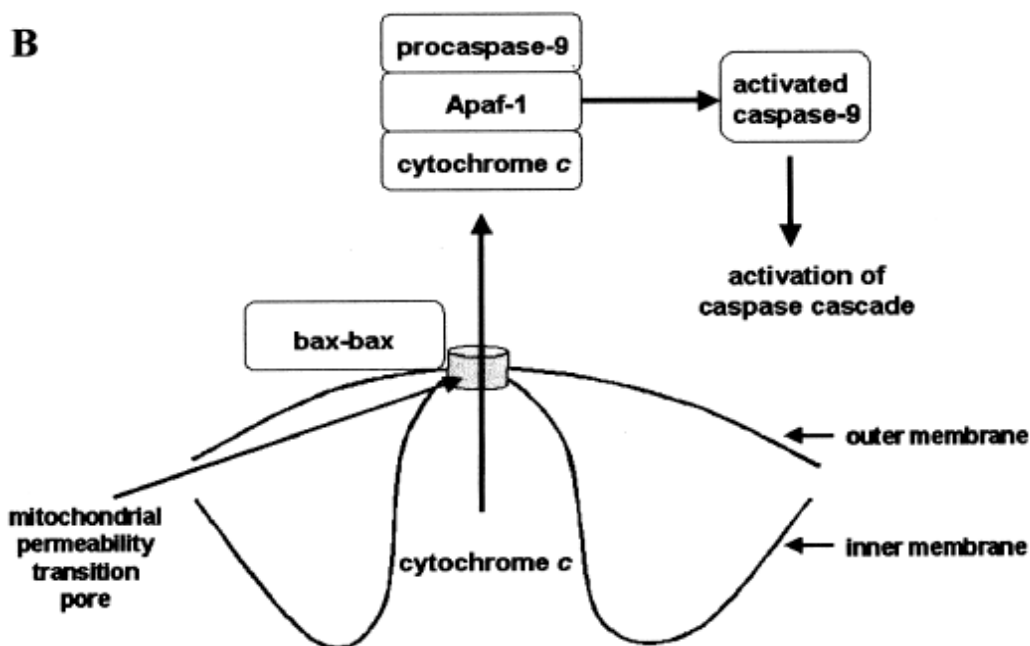
### The role of mitochondrial permeabilization

Electron microscopy has shown swollen mitochondria with ruptured outer membranes with large amounts of calcium in the matrix often in neuronal cells early after neonatal hypoxia-ischemia (Puka-Sundvall, Gajkowska et al. 2000). Increased mitochondrial permeability 0 to 1.5 hours and 6.5 to 8 hours after hypoxia-ischemia, indicating that the inner mitochondrial membrane was permeabilized to some degree after the insult, has been shown following reperfusion (Griffiths and Halestrap 1995). However, there is strong evidence that mitochondrial outer membrane permeabilization occurs during reperfusion in brain regions affected by hypoxia-ischemia (Northington, Ferriero et al. 2001, Kroemer, Galluzzi et al. 2007). The extent of apoptosis-inducing factor translocation to the nucleus correlates with the morphological distribution of neuronal injury after hypoxia-ischemia (Zhu, Qiu et al. 2003). Caspase inhibitors have also been shown to be protective in hypoxia-ischemia

(Cheng, Deshmukh et al. 1998). Apoptosis inducing factor deficiency confers considerable protection in mice subjected to neonatal hypoxia-ischemia (Zhu, Wang et al. 2007).

### **Mechanisms of Mitochondrial Permeabilization**

There are two routes that lead to mitochondrial permeabilization (Scorrano, Nicolli et al. 1997). The first one is through the opening of the permeability transition pore (PT) in the inner membrane, and it is enhanced by cyclophilin D. The second one requires a direct permeabilization of the outer membrane by Bax/Bak and is considered to be cyclophilin D-independent (Figure 1.6). It has been shown that ischemia induces opening of the cyclophilin D-dependent mitochondrial permeability transition pore in adult brain, leading to necrotic cell death (Basso, Fante et al. 2005). However, in the immature brain, Bax, rather than cyclophilin D, seems to be crucial for mitochondrial permeabilization. Bax is highly expressed in immature mitochondria. Bax translocates from cytosol to mitochondria after neonatal hypoxia-ischemia and Bax knockout mice are protected from hypoxic-ischemic brain injury (Gibson, Han et al. 2001, Northington, Ferriero et al. 2001). Therefore, Bax-dependent mitochondrial outer membrane permeabilization is a critical event in neonatal brain injury.



**Figure1.6:** Mechanisms involved in mitochondrial permeability(Banasiak, Xia et al. 2000) Permission to reproduce this figure has been granted by Elsevier.

## Genetic factors regulating cell death

The Bcl-2 family is a growing group of proteins regulating cell death. More than 15 Bcl-2 family members have been identified (Chao and Korsmeyer 1998). There are pro and anti-apoptotic proteins within the Bcl-2 family. It has been recognized for long time that pro- and anti-apoptotic Bcl-2 family proteins regulate Bax-dependent mitochondrial outer membrane permeabilization and are critical regulators of apoptotic cell death(Kroemer, Galluzzi et al. 2007). Most pro-apoptotic proteins (Bax, Bak, bok, Bad, Bim,Bid, and Puma) as well as anti-apoptotic Bcl-2 and Bcl-xL are highly expressed postnatally, followed by downregulation (except for Bcl-xL) with brain maturation (Shimohama, Fujimoto et al. 1998). Transgenic Bcl-xL overexpressing as well as Bad or Bim knockout mice are all resistant to postnatal hypoxia-ischemia(Parsadanian, Cheng et al. 1998, Ness, Harvey et al. 2006). In addition, the trophic factors insulin-like growth factor 1 (IGF-1), brain-derived neurotrophic factor (BDNF), and hexarellin, which at least partly act through

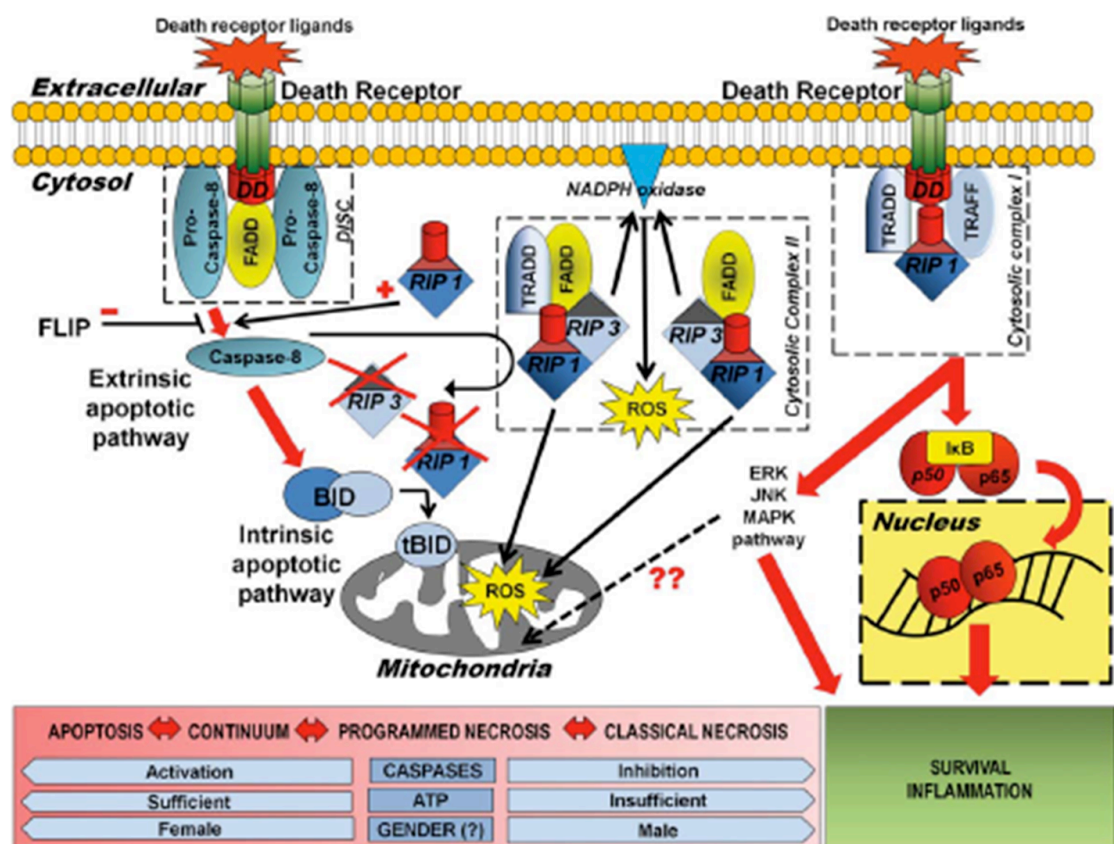
increasing anti- versus pro-apoptotic Bcl-2 family protein balance, all decrease downstream caspase activation and injury in the postnatal brain (Hagberg, Mallard et al. 2009). In summary, several Bcl-2 family proteins have an important role in neonatal brain injury.

Cell death by apoptosis can be triggered by DNA damage and p53 is involved. p53 functions in apoptosis or growth arrest and repair. Activated p53 binds the promoters of several genes encoding proteins associated with growth control and cell cycle checkpoints and apoptosis (eg, Bax, Bcl-2, Bcl-xL, and Fas) (Martin, Chen et al. 2005). p53 deficiency protects against neuronal apoptosis induced by axotomy *in vivo* and may be important in delayed neurodegeneration following neonatal HI (Martin, Kaiser et al. 2001). Acute inhibition of nuclear factor kappa B (NF- $\kappa$ B) following neonatal HI prevents both nuclear and mitochondrial accumulation of p53 while providing significant sustained neuroprotection (Nijboer, Heijnen et al. 2008).

### **Role of Caspases in cell death**

Caspases (cysteiny aspartate-specific proteinases) are cysteine proteases; 14 members have been identified so far (Wolf and Green 1999). There are three caspase-related signalling pathways have been identified that can lead to apoptosis (Figure 1.7); Firstly, the intrinsic mitochondria-mediated pathway is controlled by Bcl-2 family proteins. It is regulated by cytochrome c release from mitochondria, promoting the activation of caspase-9 through Apaf-1 and then caspase-3 activation (Li, Nijhawan et al. 1997). Caspase-3 activation results in proteolysis of essential cellular proteins, and can commit the cell to the morphological changes characteristic of apoptosis, including nuclear fragmentation (Wang, Karlsson et al. 2001). Secondly, the extrinsic death receptor pathway involves the activation of cell-surface death receptors, including Fas and tumour necrosis factor receptor,

leading to the formation of the death-inducible signalling complex (DISC) and caspase-8 activation that in turn cleaves and activates downstream caspases such as caspase-3, caspase-6, and caspase-7. Caspase-8 can also cleave Bid, leading to the translocation, oligomerization, and insertion of Bax or Bak into the mitochondrial membrane (Li, Zhu et al. 1998). The third pathway involves the activation of caspase-2 by DNA damage or ER stress as a pre-mitochondrial signal (Robertson, Enoksson et al. 2002).



**Figure1.7:** Death receptor signalling and programmed necrosis. DISC 5 “death-induced signalling complex”; FADD 5 Fas-associated death domain; HI 5 hypoxia-ischemia; IKK 5 IkappaB kinase complex; JNK 5 c-Jun N-terminal kinase; NADPH 5 nicotinamide adenine dinucleotide phosphate; NF-jB 5 nuclear factor kappa B; RIP1 5 receptor interacting protein 1; RIP3 5 receptor interacting protein 3; ROS 5 reactive oxygen species; TNF 5 tumour necrosis factor; TRADD 5 TNF receptor-associated death domain; TRAF2 5 TNF receptor-associated factor 2. (Northington, Chavez-Valdez et al. 2011) Permission to reproduce this figure has been granted by John Wiley and Sons

Caspase-3 cleavage and activation occur in brain after HI in neonatal rodents after hypoxia in neonatal piglet (Cheng, Deshmukh et al. 1998, Delivoria-Papadopoulos,



Ashraf et al. 2008). Activated caspase-3 has been shown in human post-mortem brain tissue of full term neonates with severe perinatal asphyxia. Cerebroventricular injection of a pan-caspase inhibitor or intraperitoneal injection of a serine protease inhibitor 3 hours after neonatal HI in rat has neuroprotective effects (Cheng, Deshmukh et al. 1998).

### **Excitotoxicity, nitric oxide and cell death**

As described previously, excitotoxicity and nitric oxide plays a major role in neuronal death after HI. In addition, NMDA/nitric oxide activates poly (ADP-ribose) polymerase, which depletes mitochondrial reduced nicotinamide adenine dinucleotide NAD(H) levels and triggers mitochondrial apoptosis-inducing factor release. N-methyl-D-aspartate receptor antagonists improve mitochondrial respiration, attenuate caspase-3 activation, and decrease brain injury after neonatal hypoxia-ischemia (Hagberg, Gilland et al. 1994). Furthermore, the brain protection provided by the nitric oxide synthase inhibitor 2-iminobiotin was also accompanied by near-complete inhibition of caspase-3 and reduction of apoptotic cell death (Peeters-Scholte, Koster et al. 2002).

### **Cell Surface Death Receptors**

Cell death can be initiated at the cell membrane by surface death receptors of the tumour necrosis factor receptor superfamily (TNFRSF). The signal for cell death is initiated at the cell surface by aggregation of the death domain containing members of this receptor family by their specific ligand. Clustering of the ligand on the target cell recruits Fas-associated death domain (FADD), a cytoplasmic adapter molecule that functions in the activation of the caspase 8-Bid pathway (Li, Zhu et al. 1998). Signalling for apoptosis then proceeds via the extrinsic or intrinsic pathway. Blocking Fas death receptor signalling by either pharmacologic or genetic means affords protection in these models (Graham, Sheldon et al. 2004).

## **New concepts of cell death following hypoxia-ischemia**

In neurodegeneration, cell death exists as a continuum of necrosis and apoptosis at opposite ends of a degenerative spectrum with intermediate hybrid form manifesting in between them (Figure 1.4 and 1.7). This type of cell death is observed after hypoxia-ischemia, axonal trauma and excitotoxicity in brain. Evidence for the existence of an intermediate “continuum” form of cell death has been demonstrated by the co-expression of markers for both apoptosis and necrosis in neurons in the injured forebrain at 3 hours following HI in neonatal rat. It has been shown that under certain conditions, autophagy is also part of the complex continuum of neurodegeneration following neonatal HI in mice (Ginet, Puyal et al. 2009).

It is clearly evident from the recent data that timing and energy state are critical variables in modulating autophagy or any other form of cell death following perinatal HI (Ferriero 2002). Crosstalk among multiple signalling pathways supports the concept of a cell death continuum. A fundamental cornerstone of the continuum is thought to be gradations in the responses of cells to stress. In addition, programmed necrosis is increasingly recognized as a key form of neurodegeneration and also lies along the apoptosis necrosis continuum (Northington, Chavez-Valdez et al. 2010). Necrostatin inhibits death domain kinase, which is the key intermediate in programmed necrosis. Necrostatin provides sustained neuroprotection following neonatal HI in mice (Northington, Chavez-Valdez et al. 2010).

Knowledge of these pathways of cell death has lead to a very important discovery about gender specific cell death pathways. Male neurons die mainly through activation of an apoptosis inducing factor (AIF)-dependent pathway while female neurons preferentially release cytochrome c from mitochondria and die as a result of subsequent activation of caspase 3 (Du, Hickey et al. 2009). Gender influenced the neuroprotective efficacy of 2-iminobiotin treatment – which was neuroprotective and

reduced cytochrome c release and caspase activation only in females (Nijboer, Groenendaal et al. 2007). The glutamate antagonist dextromethorphan was protective against stroke in male but not female mice at 12 days of age - this supports the greater influence of the excitotoxic pathway in immature males (Comi, Highet et al. 2006).

## **Conclusion**

In summary, neuronal degeneration in the brain exists as a continuum between apoptosis and necrosis. Hypoxia-induced neuronal apoptosis involves an ever-growing broad range of biochemical and genetic mechanisms. Many upstream events (eg, excitatory amino acids, nitric oxide, nuclear factor kappa B, and Bcl-2 family proteins) important in neonatal brain injury all contribute to mitochondrial stress during the post-hypoxia-ischemia phase and mitochondrial outer membrane permeabilization will occur, leading to irreversible cell death.

## 1.4 Animal models of Hypoxia-ischemia

Animal models have significantly contributed to our understanding of HIE in term newborn infants. The underlying pathophysiology of HIE is difficult to study in humans, therefore much of our knowledge of hypoxia-ischemia comes from animal studies. The first use of an animal model in the study of perinatal asphyxia was in 1813, when LeGallois compared the newborn rabbit's breathing with the adult animal after submersion (LeGallois 1813). He found that the respiratory efforts of newborn rabbits lasted much longer than adult animals. Subsequently, the relative resistance of newborn animal to asphyxia, when compared to adult animal, has been demonstrated in many species (Bert 1870, Britton and Kline 1945, Stafford and Weatherall 1960). Observations have also been made that males are less resistant than females, high temperature reduces resistance as does thyroxin, insulin injection, or adrenalectomy (Britton and Kline 1945). Very few animal species have escaped study, as observations have been made on the rat, cat, dog, rabbit, guinea pig, mouse, pig, sheep, monkey, baboon, and even bats.

Several areas related to perinatal asphyxia have been studied in animal models. The effects on glucose metabolism and cerebral blood flow by asphyxia have been studied in a dog model (Duffy, Cavazzuti et al. 1982). They found that glucose use was highest in brainstem structures and lowest in white matter regions. A rat model described by Rice and Vannucci, has been widely used to assess the biochemical and molecular changes occurring in the neonatal brain as a consequence of asphyxia (Rice, Vannucci et al. 1981). The model is described as a one of moderately hypoxic ischemic encephalopathy, produced by unilateral carotid artery ligation followed by 8% O<sub>2</sub> breathing at 37°C. Changes in <sup>31</sup>P nuclear magnetic resonance, metabolic rate, cerebral blood flow and EEG have been studied in the beagle puppy (Ment, Stewart et al. 1988). In sheep model, it has been demonstrated

that pre-treatment of lambs with superoxide dismutase and catalase was neuroprotective following asphyxia (Vannucci 1990), thus explained the role of free radicals in brain injury. Evidence of glutamate involvement in the pathogenesis of brain injury in hypoxia-ischemia comes from the studies performed in 1-day-old Sprague-Dawley albino rat pups (Schor 1988).

Two distinct patterns of brain injury have been described experimentally; acute total asphyxia and chronic partial asphyxia. Acute total asphyxia was induced in near term monkeys by detaching the placenta at hysterotomy (Ranck and Windle 1959). Eleven to 16 minutes later the fetuses were delivered from their membranes and resuscitated. Patterns of symmetrical injury to the ventral posterior nuclei of the thalamus, putamen and globus pallidus in the brain were found after euthanasia. These patterns were a very similar topographical distribution to that seen in infants with basal ganglia and thalamus injury following for example a sentinel event. In contrast to acute total asphyxia, eight Rhesus monkeys were studied following prolonged partial insult (Brann and Myers 1975). Intrauterine asphyxia was produced in the pregnant term monkey by breathing halothane of a high enough concentration to cause a significant drop in mean blood pressure in the mother and each fetus was exposed to asphyxia for between 1 and 5 hours. At the end of the period of asphyxia, each fetus was delivered surgically and resuscitated. Seven showed a prolonged time to first gasp until they began breathing spontaneously. Examination of brain after euthanasia showed focal cortical injury with a predominance in the posterior parietal parasagittal regions. These findings are very similar to the pattern of injury in newborn infants who have experienced chronic partial asphyxia.

Physiological, neurobiological, and pathological issues are very important when considering the relevance of experimental animals as models for brain injury in human newborn infants. The piglet models have been used to study hypoxia-ischemia by many researchers. In contrast to the newborn rodent, the percentage of

adult brain weight at birth in piglets is much closer to human (Dobbing and Sands 1979). In addition, the body size of the piglet and chest and cranial geometries, anatomy and physiology of the cardiovascular and pulmonary systems, as well as the cortical and basal ganglia topology are much more similar to human infants (Martin, Brambrink et al. 1997, Zhengyu, Zhimou et al. 2010). The basal ganglia particularly putamen and somatosensory systems, including primary somatosensory cortex and ventrobasal thalamus, are selectively vulnerable in this model following hypoxia-ischemia similar to newborn infants (Martin, Brambrink et al. 1997). Also, It has been shown that piglets and humans are comparable at birth regarding brain maturation (Grate, Golden et al. 2003). This model has also been used to study MRI and MRS effects following hypoxia-ischemia (Thoresen, Penrice et al. 1995, Penrice, Lorek et al. 1997, Munkeby, De Lange et al. 2008)

In summary, animal models of perinatal asphyxia are not ideal and will always be an approximation to the clinical situation. However, they have remarkable similarities to our current understanding of the human condition. The relevance of the animal model must be appreciated in the context of human pathobiology of the model compared to that of the human term newborn. We used piglet asphyxia model for our research because of the similarities of piglet brain to human one as mentioned above. Finally, animal models are essential for identifying relevant injury related mechanisms of HIE and for testing preclinical efficacy of therapeutics.

## **1.5 Patterns of brain injury in hypoxia-ischemia**

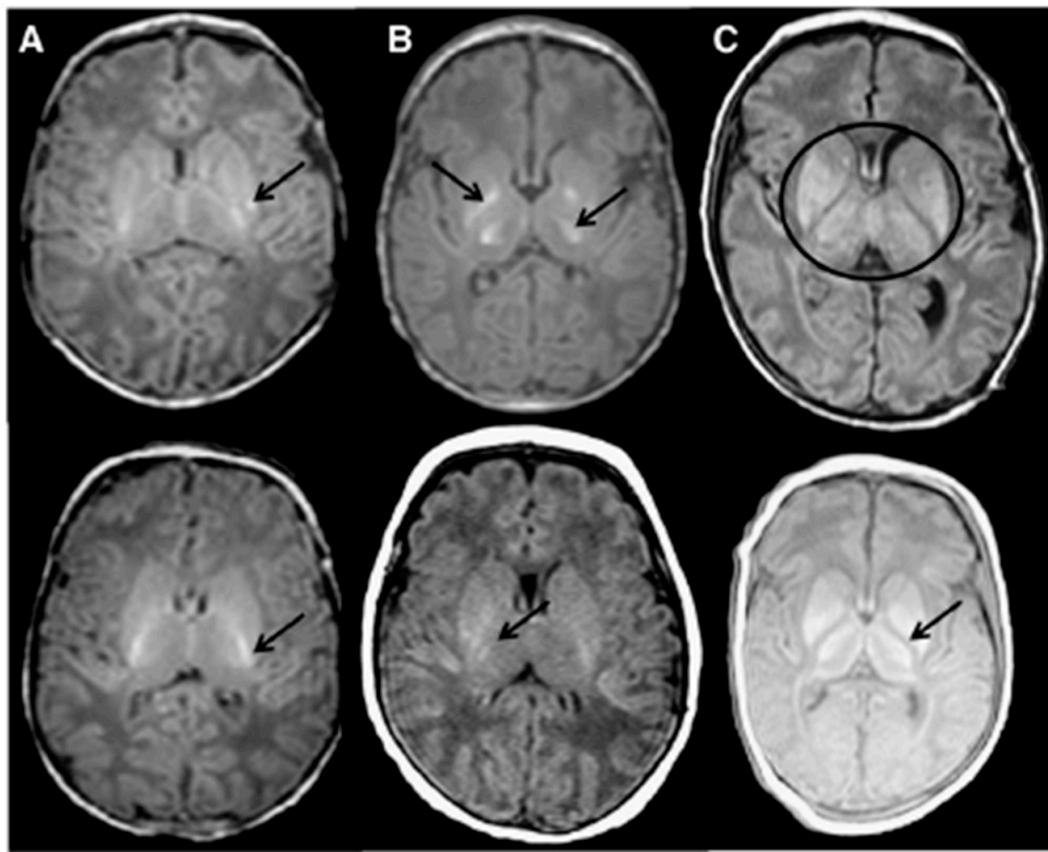
### **Introduction**

Neonatal MR imaging is invaluable in assessing the term newborn with hypoxia-ischemia. Magnetic resonance imaging provides detailed information about the pattern of lesions following perinatal brain injury and may provide excellent predictions of outcome. Patterns of brain injury are dependent on the severity and duration of the HI insult. In 1972, two patterns of perinatal brain damage was first described in an experimental model of primate fetuses, an acute and total hypoxic-ischemic event produced cerebral injury largely affecting the deep gray matter structures (basal ganglia) and prolonged but partial hypoxic-ischemic insult produced a diffuse injury to the cerebral cortex and subcortical white matter, with significantly less injury to the basal ganglia and brainstem than is seen in the setting of acute profound asphyxia(Myers 1972). Since then, many researchers have described the patterns of brain injury in term newborn infants with HIE.

### **Deep gray matter pattern**

This pattern, also called the basal ganglia and thalamus (BGT) pattern, is the most common pattern seen in infants with HIE(Martinez-Biarge, Diez-Sebastian et al. 2010). The areas affected in this pattern include ventrolateral thalami and posterior putamen and peri-rolandic cortex. These deep nuclear structures are very susceptible to acute perinatal hypoxic-ischaemic injury because of their high metabolic rate and high concentration of NMDA receptors. This pattern is seen following 'acute near total asphyxia' (Pasternak and Gorey 1998). Often there has been an acute sentinel event like a ruptured uterus, placental abruption or a prolapsed cord (Okereafor, Allsop et al. 2008). Injury to brainstem and hippocampus has also been associated with this pattern.

The intensity of resuscitation and the severity of encephalopathy are associated strongly with the basal ganglia/thalamus predominant pattern (Sie, van der Knaap et al. 2000, Miller, Ramaswamy et al. 2005). Basal ganglia and thalamic lesions were classified into mild, moderate and severe lesions depending on their extent of injury as shown in Figure 1.8(Okereafor, Allsop et al. 2008). Mild lesions are focal and subtle abnormalities in the basal ganglia and thalamus. Moderate lesions are usually multifocal lesions involving several regions of the basal ganglia. Severe lesions involve widespread abnormalities involving all BGT structures.



**Figure 1.8:** Axial T1-weighted images showing basal ganglia and PLIC abnormalities (Martinez-Biarge, Diez-Sebastian et al. 2010) Top row: A, mild basal ganglia–thalamic (BGT) lesions (arrow); B, moderate BGT injury (arrows); C, severe BGT abnormalities (circled). Bottom row: A, normal signal intensity (SI) in the posterior limb of the internal capsule (PLIC) (arrow); B, equivocal, asymmetrical and slightly reduced SI in the PLIC (arrow); and C, abnormal, absent SI in the PLIC (arrow) Permission to reproduce this figure has been granted by Elsevier.



Severe BGT lesions are often associated with the injury to the posterior limb of the internal capsule (PLIC). The internal capsule carries the major motor and sensory pathways to and from the cortex and the spinal cord. In addition, fibres from the thalamus also pass through it connecting to most regions of the cortex. It myelinates around term age, and is therefore a marker of maturation that is easily identifiable on MRI (Cowan and de Vries 2005). The internal capsule is divided into three main regions: (1) the anterior limb (ALIC) is the portion between the lentiform (lenticular) nucleus and the head of the caudate nucleus; (2) the posterior limb (PLIC) is the portion separating the lentiform nucleus from the thalamus; and (3) the genu is the portion at the junction of the above two parts. Myelination starts from the posterior limb around term gestation and progresses rostrally, complete full internal capsule by 6 – 8 months. It is very susceptible to injury at this stage of development. Depending on the myelination of PLIC, seen on MRI, it can be classified into normal, equivocal and abnormal. A normal PLIC is myelinating at term age and on MRI is seen as high signal on T1 weighted images and low signal on T2 weighted images. An abnormal PLIC has loss or reversed signal intensity on T1 and T2 weighted images (Figure 1.8). Equivocal PLIC has reduced or asymmetrical signal intensity.

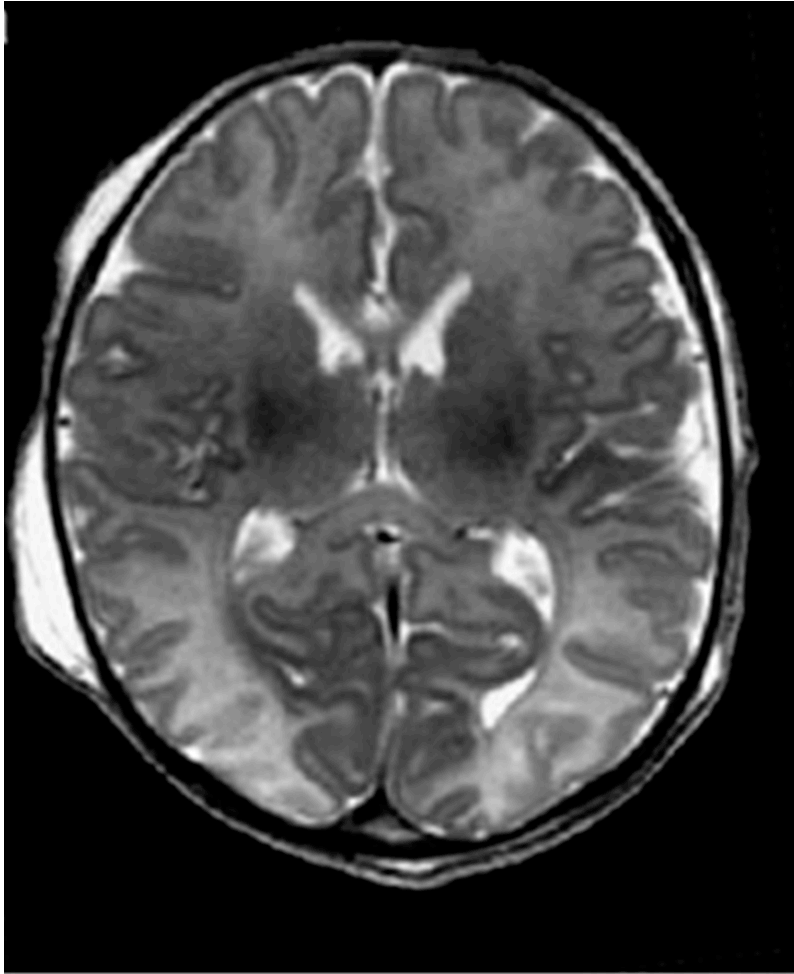
Several studies demonstrated that BGT lesions and abnormal PLIC are associated with adverse neurodevelopmental outcome. Infants with moderate to severe BGT lesions will have a range of adverse outcome including CP, seizures, cognitive impairment, feeding problems, speech and language problems, visual and hearing impairment. Cerebral palsy was the outcome observed in 10 -15% in infants with mild BGT lesions, 60 – 75% in moderate BGT lesions and 98% in severe BGT lesions (Martinez-Biarge, Diez-Sebastian et al. 2010). Around 20-30% of infants with HIE die in the neonatal period and 15-20% of children may die in the first 2-3 years. Around 50% of infants with brainstem lesions died in the neonatal or infant period (Martinez-Biarge, Diez-Sebastian et al. 2010). The signal intensity of the PLIC

is the best predictor of the ability to walk at 2 years (Martinez-Biarge, Diez-Sebastian et al. 2010).

### **Watershed predominant pattern**

This pattern is often seen following ‘prolonged partial asphyxia’. The vascular watershed zones, between anterior–middle cerebral artery and posterior–middle cerebral artery are involved, affecting white matter (Figure 1.9). This is also termed as parasagittal cerebral injury or border zone injury. The lesions can be unilateral or bilateral, posterior and/or anterior. It is also more common after hypotension, infection and hypoglycaemia, all of which may be associated with a more protracted course. The injury comprises cortical necrosis involving the immediately subjacent white matter with characteristic distribution, encompassing the parasagittal, superomedial areas of the convexities bilaterally. Later MRI may show cystic evolution, but more often atrophy and gliotic changes are noted. Another vulnerable area for ischemic cerebral injury is at the depth of the sulci as the vessels bend acutely at the cortical-white matter junction (Takashima, Armstrong et al. 1978). This area represents “a border zone within a border zone”. Therefore cerebral injury is more severe in the depths of the sulci.

Neurological signs in infants with this pattern may be mild and delayed (Sato, Hayakawa et al. 2008). They may not meet the criteria for HIE. Severe motor problems are uncommon in these infants. Commonly they have cognitive impairments including memory deficits, behavioral problems, or visual- perceptive dysfunction (Miller, Newton et al. 2002, Marlow, Rose et al. 2005). These cognitive deficits often result in delayed school-readiness and a need for additional school-age interventions. These cognitive deficits are not observed at 12 months of age, so later assessment of these infants is necessary.



**Figure1.9:** Full-term infant with watershed pattern of injury(de Vries and Groenendaal 2010) Loss of cortical ribbon is noted on the  $T_2$ weighted image, and the corpus callosum appears to be swollen with increased signal intensity. Permission to reproduce this figure has been granted by Springer.

### Other patterns

There are several other patterns of brain injury, which has been demonstrated in infants with hypoxia-ischemia. One of them is severe involvement of the subcortical white matter and cortex, with relative sparing of the immediate periventricular white matter and central grey matter. This is uncommon, and referred to as the 'white cerebrum', as diffusion weighted imaging (DWI) shows an almost completely white cerebrum, contrasted to a normal looking cerebellum (Vermeulen, Fetter et al. 2003). This condition tends to be fatal, and multicystic encephalomalacia will develop in surviving infants. Another pattern of brain injury consists of lesions restricted to the

periventricular white matter, similar to punctate white matter lesions in the preterm infant (Li, Chau et al. 2009).

In summary, there has been tremendous development of MRI techniques over the last few decades. Magnetic resonance imaging can help in detecting different patterns of brain injury in (full-term) human neonates following HIE and is thus helpful in predicting neurodevelopmental outcome.

## **1.6 Energy changes after hypoxia-ischemia, secondary energy failure and Neuroprotection**

### **Introduction**

Magnetic resonance spectroscopy (MRS) has been used to study brain energy metabolism. phosphorus-31 ( $^{31}\text{P}$ ) and proton ( $^1\text{H}$ ) MRS have provided extensive information on cerebral energy metabolism during the evolution of brain injury following hypoxia-ischemia in the newborn infant (Azzopardi, Wyatt et al. 1989), neonatal rat (Blumberg, Cady et al. 1997) and the newborn piglet (Lorek, Takei et al. 1994).

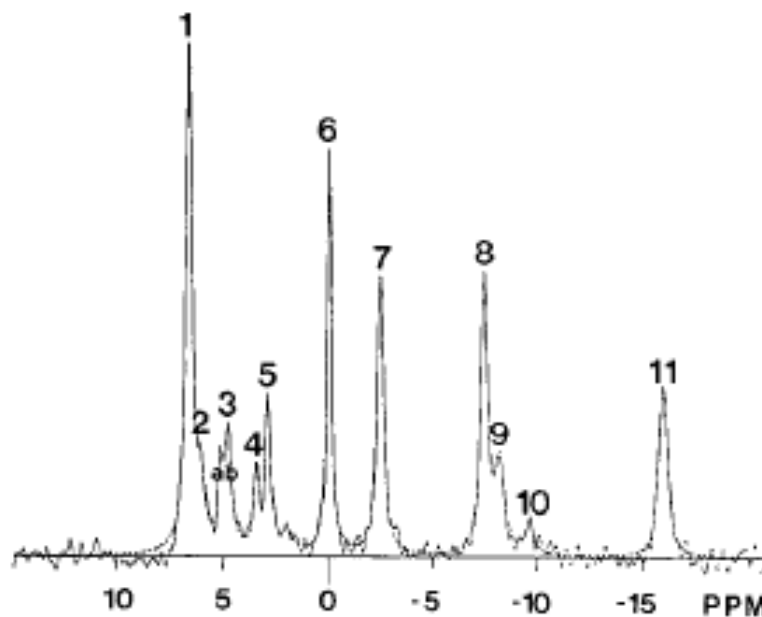
### **Initial energy changes**

During a hypoxic-ischemic insult, there is a profound failure in cerebral energetics which resolves rapidly with resuscitation and reperfusion. Severe hypoxia-ischemia rapidly results in cessation of cerebral oxidative metabolism, accumulation of cerebral lactate and rapid depletion of adenosine tri-phosphate (ATP). This decrease in ATP triggers a series of additional mechanisms that begin with failure of the ATP-dependent Na/K pump (Volpe 2001) (as discussed above). Increased lactate occurs which is due to reduced oxygen supply resulting in decreased oxidative phosphorylation, causing enhanced glycolysis via the Pasteur effect. Initial lactate accumulation could be beneficial as it can act as a fuel and leads to generation of ATP from phosphocreatine (PCr)(Schurr 2006). However, severe tissue acidosis, which develops later, results in neuronal injury.

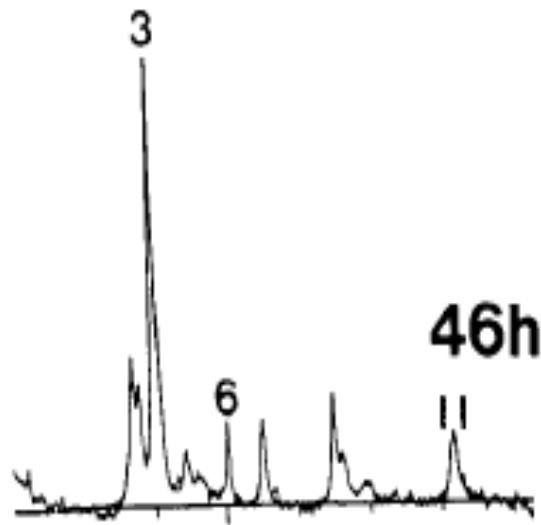
### **Delayed or Secondary energy failure**

Initial energy changes noted during HI ( $^{31}\text{P}$  MRS) return to normal levels after resuscitation in human infants (Hope, Costello et al. 1984, Lorek, Takei et al. 1994). However, several hours later, there follows a progressive decline in phosphocreatine

(PCr) and ATP, increase in inorganic phosphate (Pi) associated with a rise in lactate despite adequate resuscitation (Hope, Costello et al. 1984, Cady, Amess et al. 1997)(Figures 1.10, 1.11 and 1.12). These changes were termed delayed or secondary energy failure (SEF) to distinguish them from the primary energy failure that occurs during the acute hypoxic-ischemic insult. This SEF was observed only in infants with adverse outcome (Azzopardi, Wyatt et al. 1989). Thus, SEF was consequential to a pathological mechanism initiated by intrapartum HI or/and reperfusion.

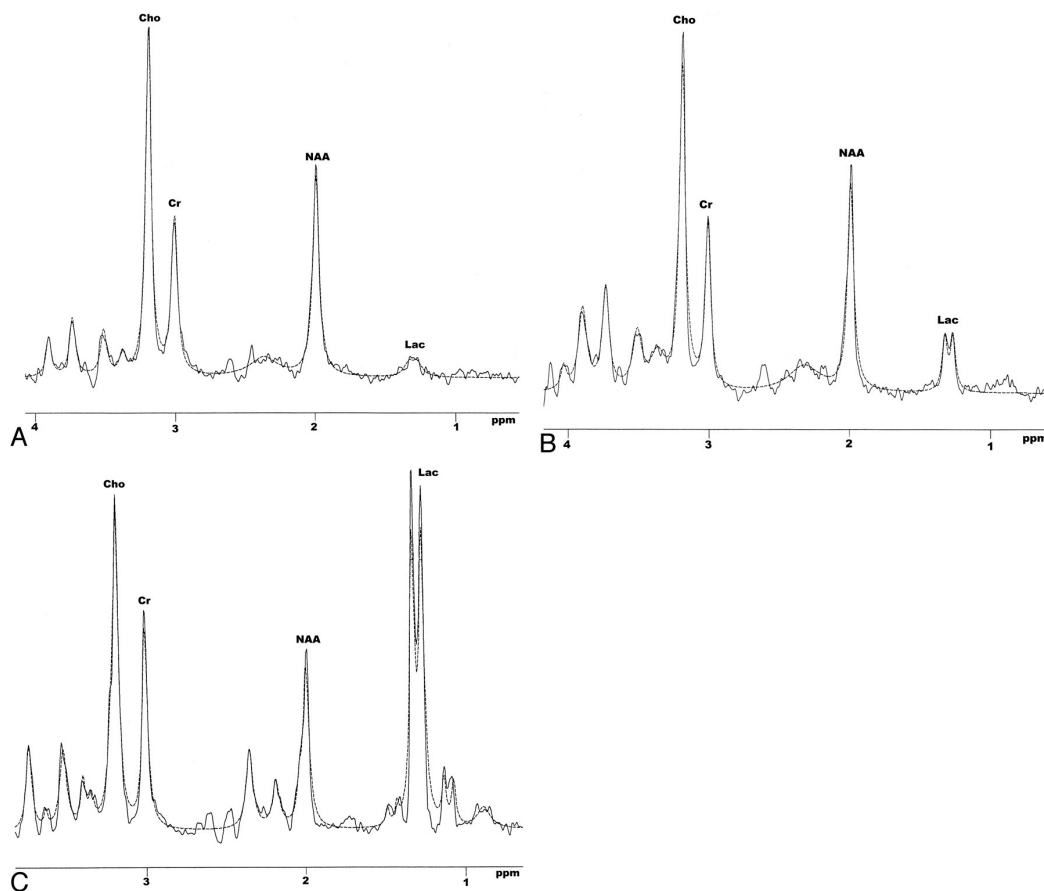


**Figure 1.10:**  $^{31}\text{P}$  spectrum from a control animal (Lorek, Takei et al. 1994) 1, phosphoethanolamine; 2, phosphocholine; 3a and b, Pi components; 4, phosphotidyl ethanolamine; 5, phosphotidyl choline; 6, PCr; 7,  $\gamma$ -NTP; 8,  $\alpha$ -NTP; 9, NAD and NADH; 10, uridine diphosphate sugars; 11,  $\beta$ -NTP. Permission to reproduce this figure has been granted by Nature Publishing Group.



**Figure 1.11:**  $^{31}\text{P}$  spectrum from an animal, acquired at 46 hours after 43 minutes of HI. (Lorek, Takei et al. 1994) Permission to reproduce this figure has been granted by Nature Publishing Group.

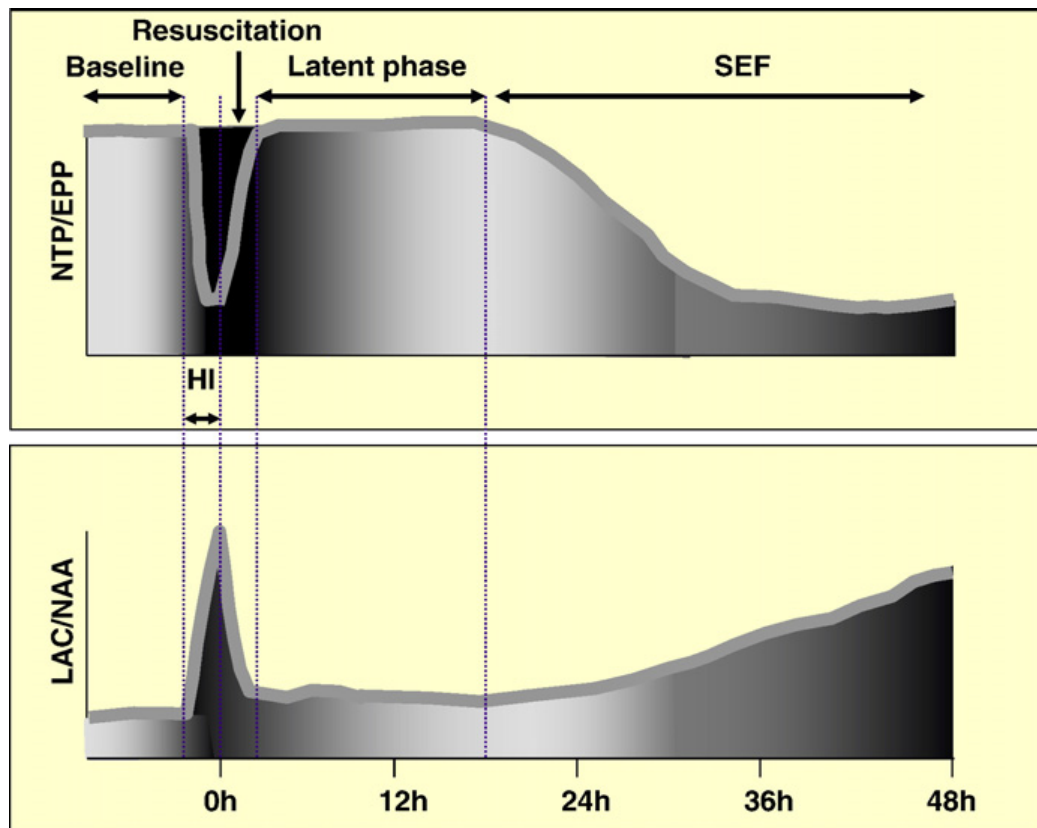
Explanations for the delayed rise in cerebral lactate include inhibition of mitochondrial respiration or irreversible mitochondrial damage. Increased lactate correlate closely with falling nucleotide triphosphate (NTP)/exchangeable phosphate pool ( $\text{EPP} = \text{Pi} + \text{PCr} + \text{NTP}$ ), and a possible explanation for this is that a fall in ATP concentration is required before the glycolytic enzymes phosphofructokinase and pyruvate kinase are significantly activated (Penrice, Lorek et al. 1997). Another factor to the delayed rise in lactate could be the removal of dead or dying cells by phagocytosis. The activation of human monocytes and macrophages enhances glycolysis leading to increased lactate production (Lopez-Villegas, Lenkinski et al. 1995).



**Figure 1.12:** Representative  $^1\text{H}$  MRS brain spectra from a control infant and 2 neonates with neonatal encephalopathy (Cheong, Cady et al. 2006). This was acquired between 2 and 4 days of age from an 8-mL voxel centred on the thalami by using PRESS (TE, 270 ms; and TR, 2 seconds). The dashed lines are the spectrum analysis Lorentzian profiles fitted to the peaks. A, Control; B, Normal/mild outcome; C, Severe/fatal outcome. Permission to reproduce this figure has been granted by American Society of Neuroradiology.

The biphasic decrease in NTP/EPP and reciprocal increase in brain lactate during and following a transient hypoxic–ischaemic insult are shown in figure 1.13. The mechanisms behind the progression from primary energy failure to secondary energy failure are those involving excitotoxins which results in cell death as explained above. In addition, the severity of secondary energy failure depends on the severity on primary insult (Lorek, Takei et al. 1994). The knowledge from these studies provided a basis for the realization that rescue treatment after hypoxia-ischemia may reverse or ameliorate SEF. Experimental studies with moderate hypothermia and other therapeutic agents soon followed (Thoresen, Penrice et al. 1995).





**Figure 1.13:** The biphasic pattern of energy failure associated with a transient HI insult (Kelen and Robertson 2010).  $^{31}\text{P}$  MRS (top) and  $^1\text{H}$  MRS (bottom) in the UCL piglet model.  $^{31}\text{P}$  MRS: nucleotide tri-phosphate (NTP)/exchangeable phosphate pool (EPP=Pi+PCr+NTP) is shown on the y axis. The change in NTP/EPP during transient hypoxia–ischaemia (HI), resuscitation, the latent phase (period between the recovery from acute HI and the evolution of secondary energy failure (SEF)) and SEF itself are shown.  $^1\text{H}$  MRS: lactate/N acetyl aspartate (NAA) is shown on the y axis. The change in Lac/NAA during transient HI, resuscitation, latent phase and SEF are shown. Permission to reproduce this figure has been granted by Elsevier.

## Therapeutic hypothermia

Experimental and clinical data indicate that induced hypothermia reduces cerebral hypoxic-ischemic injury. A series of large randomized clinical trials have now shown that moderate hypothermia improves neurological outcome at 18 months in infants with HIE (Gluckman, Wyatt et al. 2005, Shankaran, Laptook et al. 2005, Azzopardi, Strohm et al. 2009). Meta-analysis of all trials with 18 months of follow-up suggests a number needed to treat of approximately 9, with a relative risk for death or disability of 0.81 (95% confidence interval, 0.71–0.93;  $p < 0.002$ ) (Edwards, Brocklehurst et al. 2010). Therapeutic hypothermia is now widely offered to infants with NE in developed countries (Kapetanakis, Azzopardi et al. 2009). Recently in the UK, the National

Institute for Health and Clinical Excellence (NICE) recommended that therapeutic hypothermia for Hypoxic Perinatal Brain Injury should be offered to 'carefully selected infants' (NICE 2010).

There are many neuroprotective actions of hypothermia after HI. Cerebral metabolism is approximately reduced 7% for every 1°C drop in core temperature, with consequently lower glucose and lower oxygen demand (Erecinska, Thoresen et al. 2003). Hypothermia reduces free radical production and glutamate levels, thereby preserves mitochondrial function (Gunn and Gunn 1998). It also decreases apoptosis by inhibiting caspase 3 activation and decreasing Bcl-2 expression (Edwards, Yue et al. 1995).

The safety aspect of therapeutic hypothermia is well established from all clinical trials. Thrombocytopenia and arrhythmias are frequently associated with therapeutic hypothermia (Shah 2010), but these can be corrected in the setting of an experienced neonatal intensive care unit. Use of ionotropes was also observed in some studies with hypothermia; however, they are due to physician bias rather than actual need (Battin, Thoresen et al. 2009). In addition, there is a need to re-warm as slowly as possible – by a maximum of 0.5°C over 2 hours, because adverse effects can occur during rewarming (Battin, Bennet et al. 2004). Furthermore, it is very important to avoid over-cooling below the recommended temperature, which has been associated with adverse outcomes (Rohrer and Natale 1992, Sessler 2001, Polderman 2004)

### **Adjunct therapies**

Approximately half the infants with HIE, who receive therapeutic hypothermia, still have an abnormal outcome (Edwards, Brocklehurst et al. 2010). Evidence from animal studies suggests that hypothermia extends the duration of the therapeutic window and certain drugs given during this time may augment neuroprotection (Liu,

Barks et al. 2004). Research is now being focused on pre-clinical studies of drugs, which act synergistically or additively with hypothermia. Promising agents include erythropoietin, N acetyl cysteine (NAC), melatonin, 2-immunobiotin and inhaled xenon (Kelen and Robertson 2010).

## **1.7 Cerebral Magnetic Resonance Spectroscopy**

### **Biomarkers**

#### **Introduction**

A National Institutes of Health working group defined biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Atkinson AJ 2001). Bridging biomarkers are the ones that detect an effect in both animals and humans and they allow the transfer of information from experimental to human studies. As research is now being focused on preclinical and clinical studies of neuroprotective drugs, which acts synergistically with therapeutic hypothermia, early biomarkers are urgently needed to select the infants, at risk of an abnormal outcome, for neuroprotective intervention. Late biomarkers are also needed to prognosticate long term neurodevelopmental outcome following these neuroprotective interventions to avoid the delay between intervention and long-term outcome. Magnetic resonance spectroscopy (MRS) can provide valuable information on cerebral energy metabolism following hypoxia-ischemia that can be used as early or late biomarkers.

#### **Proton magnetic resonance spectroscopy ( $^1\text{H}$ MRS) Biomarkers**

Several MRS studies have used  $^1\text{H}$  MRS because the greater sensitivity of the  $^1\text{H}$  nucleus allows more accurate regional measurements. Within the  $^1\text{H}$  spectrum, peaks can be assigned using appropriate scanning parameters to N-acetyl aspartate (NAA), choline-containing compounds (Cho), creatine plus phosphocreatinine (Cr), lactate (Lac), myoinositol (ml) and alanine, glutamine and glutamate (Glx). In  $^1\text{H}$  MRS, the NAA resonance is a singlet and can be observed easily as a peak with a chemical shift of 2.02 ppm over a wide echo-time (TE) range (Figure 1.12). However,

the cerebral Lac resonance visualized easiest is the methyl doublet with J coupling of ~6.93 Hz(Kingsley 1994): as a result the Lac doublet undergoes phase modulation and point-resolved spectroscopy (PRESS; an often-used single-voxel localization technique) detection is optimized for TE 144 ms (inverted signal) and TE 288 ms (upright signal)(Ernst and Hennig 1991). The Lac doublet is observed at 1.33ppm (Figure 1.12).

Cerebral Lac rises during hypoxia-ischemia and normalises soon after resuscitation and reperfusion(Lei and Peeling 1998). There is a secondary, delayed rise in Lac several hours after hypoxia-ischemia due to renewed production or impaired mitochondrial utilization (Penrice, Lorek et al. 1997). This secondary Lac rise can persist for a long time (Hanrahan, Cox et al. 1998) and is associated with brain alkalosis (Robertson, Cowan et al. 2002). The presence of Lac is thought to reflect neural injury as well as the inflammatory reaction to damage through microglia activation(Garcia and Kamiyo 1974). NAA is unique not only by virtue of its exceptionally high concentration in the brain, but also due to the powerful signal that it gives off in water-suppressed proton MRS spectrograms(Luyten and den Hollander 1986). Evidence from several animal studies shows that decrease in NAA occur more slowly than Lac increases after focal ischemia(Barker P 2005). The lowest levels of NAA in animal studies are observed 50–70 hours after hypoxia-ischemia(Higuchi, Fernandez et al. 1996). Large declines in NAA levels following hypoxia-ischemia parallel the onset of reduced neuronal numbers, reduced cell size, nuclear pyknosis and infiltration first of polymorphonuclear cells and mononuclear cells, which are most evident between 24 and 48 hours in focal lesions in rats (Lee, Burdett et al. 1996). A rise in myo-inositol (marker for glia) and glutamate may also be observed following hypoxia-ischemia with scanning parameters optimised to demonstrate these peaks(Groenendaal, Roelants-Van Rijn et al. 2001, Robertson, Lewis et al. 2001).

Over the last two decades, several clinical studies used these markers to predict neurodevelopmental outcome. Although absolute concentrations can be obtained, most studies measured metabolite ratios including Lac/NAA, Lac/Cr, NAA/Cho, NAA/Cr and Lac/Cho from peak areas. These studies have shown a significant association between a number of metabolite ratios and neurological outcome following asphyxia. A recent meta-analysis, which included all these studies, suggested that deep gray matter Lac/NAA, acquired between 5-14 days after birth, was the most accurate predictor of long term adverse neurodevelopmental outcome with a sensitivity of 0.82 (95% CI:0.74-0.89) and specificity 0.95 (0.88-0.99) (area under curve (AUC) 0.94 [SE 0.03])(Thayyil, Chandrasekaran et al. 2010). The median cut-off values of all the biomarkers are in table 1.4.

MRS measurement	Threshold	Sensitivity	Specificity
Lac/NAA	0.29 (0.24–0.4)	0.82 (0.74–0.89)	0.95 (0.88–0.99)
Lac/Cr	0.39 (0.32–0.95)	0.77 (0.64–0.86)	0.94 (0.85–0.98)
Lac/Cho	0.25 (0.2–0.25)	0.84 (0.71–0.93)	0.81 (0.65–0.91)
NAA/Cho	0.72 (0.6–1.0)	0.59 (0.46–0.71)	0.72 (0.50–0.83)
NAA/Cr	1.2 (0.84–1.45)	0.61 (0.51–0.70)	0.71 (0.61–0.80)

All values are median (range).

Lac, lactate; NAA, *N*-acetyl aspartate; Cr, creatine plus phosphocreatinine; Cho, choline-containing compounds.

**Table 1.4:** The median cut-off values of all  $^1\text{H}$  MRS biomarkers(Thayyil, Chandrasekaran et al. 2010) Permission to reproduce this table has been granted by American Academy of Pediatrics.

### Phosphorus magnetic resonance spectroscopy ( $^{31}\text{P}$ MRS) biomarkers

Phosphorus ( $^{31}\text{P}$ ) MRS defined the biphasic pattern of cerebral energy metabolism in infants with HIE, which is impairment of cerebral energy metabolism following several hours after birth despite adequate resuscitation in moderate to severe encephalopathic infants (Hope, Costello et al. 1984, Azzopardi, Wyatt et al. 1989). During transient hypoxia-ischemia in experimental models, similar biphasic cerebral energy metabolism impairments have been seen with decrease in PCr, NTP, and intracellular pH, and increase in Pi, but these rapidly return towards basal values on resuscitation followed by a "latent period" of apparently almost normal energetics (Lorek, Takei et al. 1994, Vannucci, Towfighi et al. 2004). Then, 12 to 24 hours later,

a fall in PCr and NTP, and increase in Pi was observed but these changes are unaccompanied by profound intracellular acidosis.

Clinical studies using  $^{31}\text{P}$  MRS demonstrated a close relationship between severity of impairment in cerebral energy metabolism and adverse neurodevelopmental outcome (Azzopardi, Wyatt et al. 1989, Roth, Edwards et al. 1992). Following hypoxia-ischemia reduced cerebral PCr/Pi and NTP/total mobile phosphate ( $\text{P}_{\text{tot}}$ ) ratios, and low PCr, NTP, and  $\text{P}_{\text{tot}}$  concentrations have been associated with eventual neurodevelopmental impairment and increased infant mortality (Azzopardi, Wyatt et al. 1989, Martin, Buchli et al. 1996). In particular, reduced NTP/ $\text{P}_{\text{tot}}$  was invariably associated with fatal outcome. Thus in clinical studies, PCr/Pi and NTP/ $\text{P}_{\text{tot}}$ , and PCr, NTP, and  $\text{P}_{\text{tot}}$  concentrations have proven useful biomarkers.

## **Conclusion**

In summary, the cerebral  $^1\text{H}$  MRS Lac/NAA peak area ratio is the most accurate quantitative MR biomarker for prediction of neurodevelopmental outcome. However,  $^{31}\text{P}$  and  $^1\text{H}$  MRS have not yet been shown to be effective biomarkers of treatment effect in human infants. Further validation of all MRS biomarkers will be important for monitoring neuroprotective therapies and providing surrogate endpoints for future Phase II clinical trials of combinations of adjuvant drugs and therapeutic hypothermia.

## 1.8 Aim and Hypotheses

To explore the precise role of MRS as a biomarker in perinatal hypoxia-ischemia, further studies are needed to assess the relation between MRS and outcome measures such as neuronal cell death and/or neurodevelopmental outcome. It is also essential to determine the effect of neuroprotective treatments on MRS and whether similar relationships between MRS and outcome measures exist. We have used the piglet model to test the following hypotheses.

Hypothesis 1: Early  $^1\text{H}$  MRS and  $^{31}\text{P}$  MRS biomarkers (EBMs), acquired within first 4 hours after global hypoxia-ischemia, correlate with neuronal injury assessed at 48 hours after hypoxia-ischemia in the piglet asphyxia model.

Currently, therapeutic hypothermia should be started for infants with NE as soon as possible after diagnosis, usually within 6 hours of birth (NICE 2010). Hence there is a short period (termed as therapeutic window) available for diagnosis before starting any neuroprotective treatment. During this therapeutic window, infants are selected to receive therapeutic hypothermia on the basis of history of events related to birth, abnormal clinical examination, presence of seizures and abnormal amplitude integrated EEG. However, there is no perfect tool available early to detect possible short-term outcome or ideal candidate for neuroprotective intervention. During this therapeutic window, MRS could be performed, once the infant is stabilized, to predict the outcome of the baby before starting neuroprotection. Hence we formulated this hypothesis to identify whether early MRS biomarkers can predict neuronal injury assessed at 48 hours by MRS and histology.

Hypothesis 2: Late  $^1\text{H}$  MRS and  $^{31}\text{P}$  MRS biomarkers, acquired at 48 hours of hypoxia-ischemia, correlate with neuronal cell death assessed at 48 hours after hypoxia-ischemia in the piglet asphyxia model; this holds true with and without



neuroprotective intervention. The two neuroprotective interventions we describe are Xenon/hypothermia (Faulkner, Bainbridge et al. 2011) and N-Methyl-Isobutyl-Amiloride (MIA) (Robertson, Kato et al. 2013).

Over the last two decades, several clinical studies used  $^{31}\text{P}$  and  $^1\text{H}$  MRS to predict neurodevelopmental outcome in infants with NE. These clinical studies demonstrated a close relationship between metabolite ratios ( $^1\text{H}$  MRS) /severity of impairment in cerebral energy metabolism ( $^{31}\text{P}$  MRS) and adverse neurodevelopmental outcome. However,  $^{31}\text{P}$  and  $^1\text{H}$  MRS have not yet been shown to be effective biomarkers of treatment effect in human infants. Further validation of all MRS biomarkers will be important for providing surrogate endpoints for future clinical trials of combinations of adjuvant drugs and therapeutic hypothermia. Hence we formulated this hypothesis to assess the predictive ability of MRS biomarkers after neuroprotective intervention.

Hypothesis 3: We have extended hypothesis 2 in a clinical cohort of infants at UCH. In a clinical cohort of infants with neonatal encephalopathy (NE), half of whom received hypothermic neuroprotection,  $^1\text{H}$  MRS biomarkers, acquired between 5 and 14 days after birth, predict neurodevelopmental outcome at 18 months age.

## **Chapter 2 - Materials and Methods**

## **Animals**

Large white male piglets (1.6 – 2.0 Kg) aged < 24hrs were used for all experiments. Experiments were performed under UK Home Office Guidelines (Animals Scientific Procedures Act 1986) and with the project licence (PPL 70/6355).

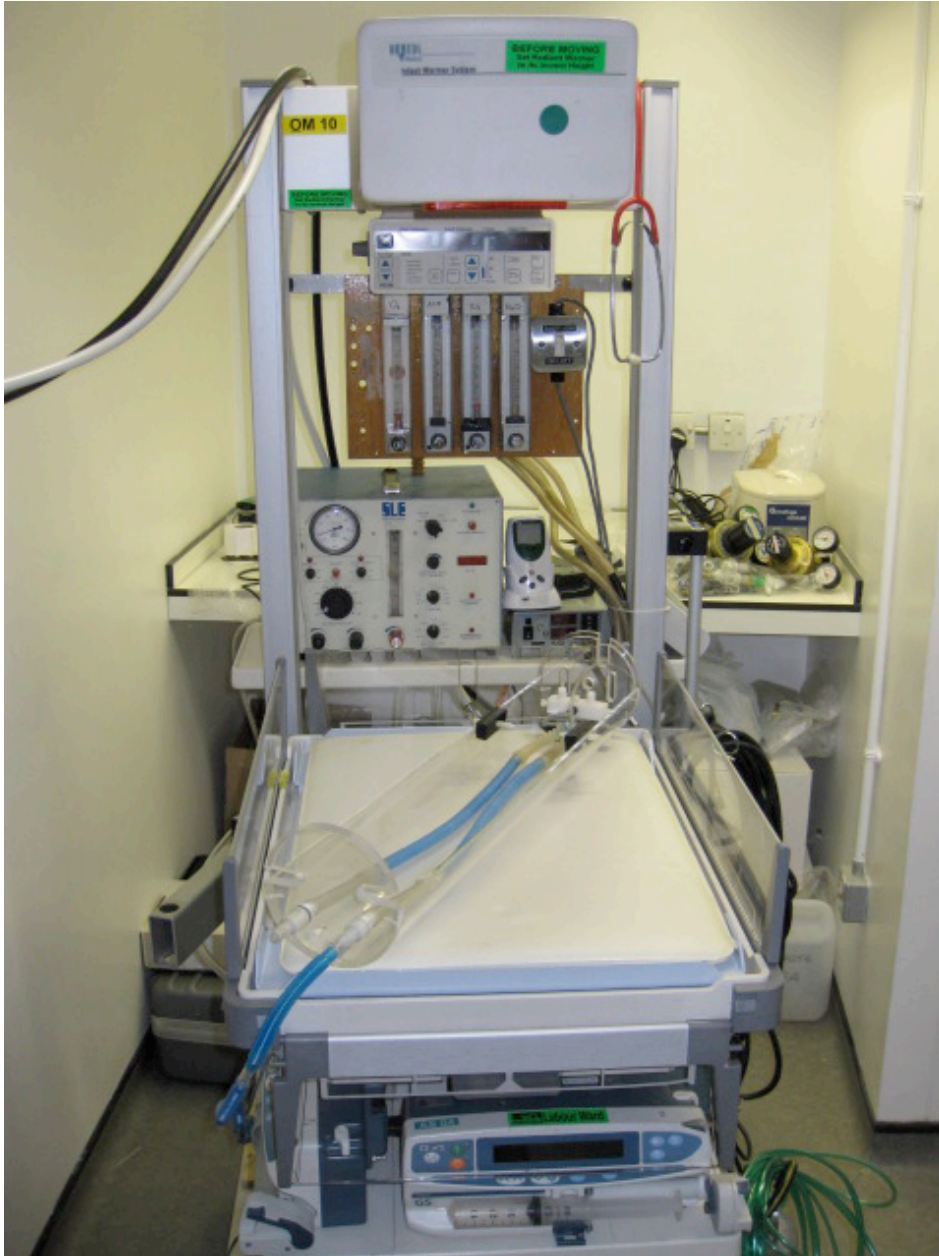
## **Initial preparation**

On the morning of the experiment, the piglet was sedated on arrival with intramuscular midazolam (0.2mg/kg) and moved to a resuscitaire (Figure 2.1) for surgical procedures. Isoflurane anesthesia (initial concentration 4% (v/v)) was given through a facemask to induce anaesthesia. The piglet was laid supine with its head immobilised and all limbs loosely restrained with string. Isoflurane anaesthesia was then reduced to 3% for surgery. Arterial oxygen saturations and heart rate were monitored with pulse oximeter (NoninMedical,USA) and O<sub>2</sub> saturations maintained above 92%. The rectal temperature was maintained at normothermia (38.5 °C +/- 0.5 °C) with the help of an overhead heater.

## **Tracheostomy**

The skin site was cleaned with povidine iodine and an incision was made with a sterile scalpel blade between top of rib cage and the area just below the laryngeal prominence. Using blunt forceps, the skin, muscle and connective tissue were gently resected in a vertical direction. The trachea was located and isolated from surrounding connective tissue and two lengths (10cm) of Mersilk suture were pulled through underneath the trachea. These suture threads were separated and positioned at either end of the isolated trachea. The trachea was cut to 50% of its depth (180° cut) between the cartilage rings. The endotracheal (ET) tube (3.5-4mm inner diameter) was inserted swiftly into the tracheal opening and the depth of the tube maintained around 2.5cm. The ventilator circuit was attached to the ET tube and continuous mandatory ventilation (CMV) mode of ventilation with a peak inspiratory pressure (PIP) of 15cmH<sub>2</sub>O, positive end expiratory pressure (PEEP) of 4cmH<sub>2</sub>O and

rate of 12/min. The ET tube was secured anterior and posterior of the incision point with the sutures. The ventilator settings were adjusted to maintain partial pressure of oxygen ( $\text{PaO}_2$ ) and carbon dioxide ( $\text{PaCO}_2$ ) at 8-13 kPa and 4.5-6.5 kPa, respectively.



**Figure2.1:** Resuscitaire and pod

### **Carotid artery occluder placement**

Once the tracheostomy was completed and the animal stable on mechanical ventilation, the carotid artery was located on one side of trachea and gently dissected with forceps from the surrounding tissue and nerve at the level fourth cervical vertebra. Landmarks for the artery were medial to the trachea with the vein lateral and the nerve beneath. Once isolated, the artery was elevated with forceps a vascular occlude (OC2A, In Vivo Metric, Healdsburg, California, USA.) was placed around the artery ensuring cuff size and artery diameter were matched closely. A suture was passed through the fixation holes in the cuff and the artery was released within 1 minute to lie in its original position. The first knot was tied firmly and the second knot loosely to hold the occlude in position. The cuff was manually inflated to check for successful inflation, to ensure complete vessel occlusion, and to ensure the return of normal pulsations when the artery was released. Following this the second knot was fixed tightly and the double knot repeated. The artery was relocated to its original position within the surrounding tissue, ensuring that the artery direction was vertical and the cuff lay perpendicular to this. The same procedure was repeated on the other carotid artery. The skin was closed using Mersilk sutures and 'return suture' technique ensuring no restriction of the occluder-tubing occurred.

### **Umbilical vessel catheterisation**

For the umbilical arterial catheterisation, a single lumen catheter (3.5Fr) was attached to a 3-way tap followed by an extension tube. For venous catheterisation, a double lumen catheter (3.5Fr) was used with a 3-way tap on each end. The skin site was cleaned with povidine iodine. The umbilical cord was removed with a sterile scalpel at the boundary of live/dead tissue. Umbilical vessels were identified (2 arteries-smaller and thin walled; 1 vein- larger, thick walled) and isolated. The artery was dilated with the inset tip of thin tip forceps, 3-5 mm down the vessel. Holding the edge of the vessel with forceps, a single lumen catheter was inserted into the artery

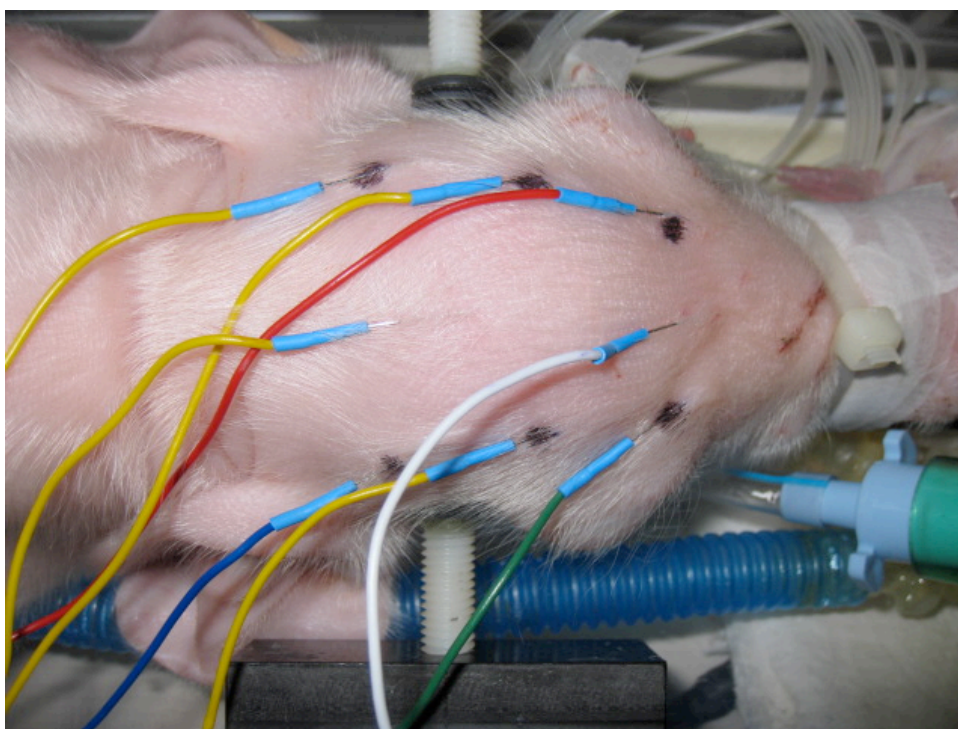
until a depth of approx. 7-9cm was achieved. Once blood flow was observed, the catheter was secured by suturing the surrounding skin and to the catheter with 3 x knotted sutures. The same procedure was repeated for catheterising the umbilical vein, leaving the catheter 5cm from the skin surface. The catheter lines were taped together then both catheters were taped in a loop to the lower abdominal region. When difficulties with umbilical vessel catheterisation were encountered, the femoral artery and vein were catheterized instead using 22-20G intravenous catheters.

### **Locating animal into pod**

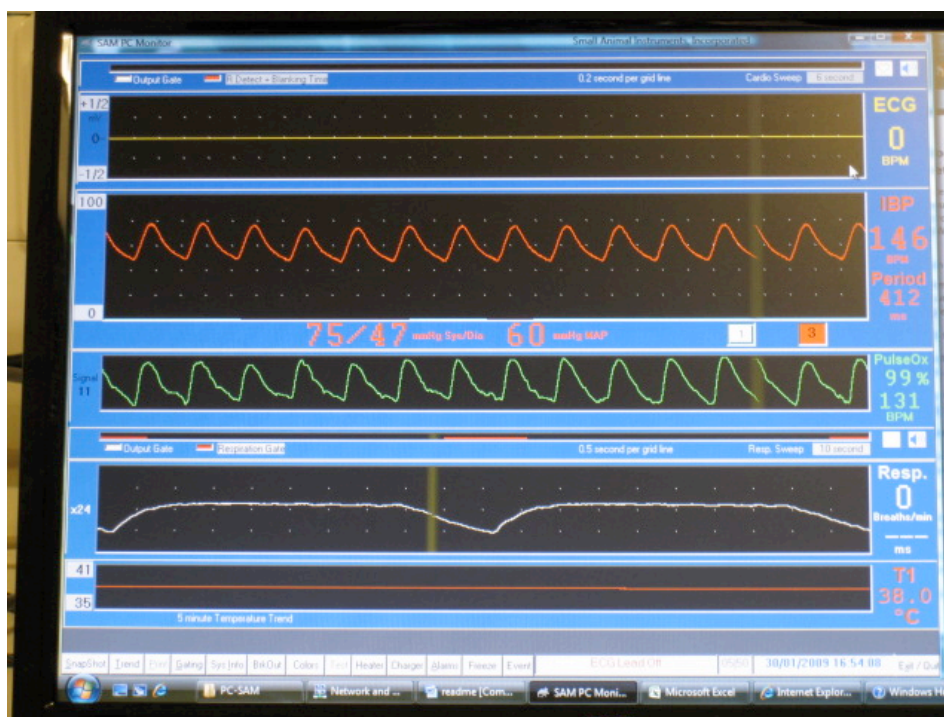
After surgery, the piglets were positioned prone in a plastic pod. A bite bar was located in the mouth and hooked onto the upper canines. It was secured with tape and fixed firmly with a cable tie. The animal was placed prone, the head was secured into a stereotactic frame and ear bars were firmly positioned against the temple (Figure 2.2). All tubes, catheters and cables were carefully arranged. The animal remained in the pod until the end of the experiment.

### **Monitoring**

All animals received continuous physiological monitoring and intensive life support throughout the experiment. Continuous heart rate, ECG, arterial oxygen saturations, arterial blood pressure, rectal temperature was recorded continuously (SA instruments, NY, USA) (Figure 2.3). Intermittent blood sampling was performed from the umbilical arterial line and  $\text{PaO}_2$ ,  $\text{PaCO}_2$ , pH, electrolytes, glucose, and lactate (Abbot Laboratories, UK) were measured 6hrsly and temperature corrected (Abbott istat, UK). Trace EEG and aEEG were monitored continuously whenever the animal was outside the 4.7 T magnet bore with a Brainz CFM monitor.



**Figure 2.2:** Animal in the pod, ventilated via tracheostomy and needle electrodes for EEG



**Figure 2.3:** Vital signs including HR, BP, Oxygen saturations and temperature monitoring during the experiment

## **Fluids and Drugs**

Maintenance fluids (10% dextrose, 60ml/kg/day) were infused through the umbilical venous catheter throughout the experiment. Antibiotics (Benzyl penicillin 50mg/kg and gentamicin 2.5mg/kg, every 12 h) were administered intravenously. Arterial lines were maintained by infusing 0.9% saline solution (Baxter, 1ml/h): heparin sodium was added at a concentration of 1 IU/ml to prevent line blockage. Bolus infusions of colloid (Gelofusin, B Braun Medical Ltd. Emmenbrucke, Switzerland) and ionotropes including dopamine(5-20µg/kg/min) dobutamine(5-20µg/kg/min) and adrenaline (0.1 – 120µg/kg/min) maintained mean arterial blood pressure (MABP) >40mmHg. Seizures, whenever observed, were treated with phenobarbitone (20mg/kg repeated if necessary).

## **Pain control**

Isoflurane anesthesia (initial concentration 4% (v/v)) was administered during surgery. The Isoflurane concentration was reduced following the initial surgical procedures and thereafter kept at around 2%. A continuous fentanyl infusion (3 – 6µg/kg/hour) was given through the umbilical venous catheter. Adequate sedation and analgesia were maintained throughout the experiment.

## **Termination and Perfusion**

Forty-eight hours after the hypoxic-ischemic insult, the animal was euthanized with pentobarbital (6ml) followed by saline (5ml) bolus, both given through the umbilical venous line. The animal was laid supine in a tray so its head was in line with its body. Carotid artery occluders were first removed; an incision was made along the midline of the chest with a scalpel starting from manubrium sterni and then laterally following the underside of the rib cage to form an inverted 'Y'. The skin overlying the rib cage was separated with the blunt end of forceps. Using large blunt scissors, the thoracic cavity was punctured at the base of the rib cage and cut medial to lateral and anterior, towards the shoulder through the rib cage on each side. Parting the



connective tissue exposed the heart. The rib cage was cut open at the sternum and folded back towards head holding it in place with a heavy pair of surgical clamps.

Once the heart was fully exposed, a small incision was made in the left ventricle and a small catheter was passed through the incised hole the point of the catheter reached the aortic arch. Then PBS was slowly infused (approximately 50mls over 1min) through the catheter with a syringe, ensuring that there were no air bubbles in the syringe. A small incision was made in the right atrium additionally to ensure blood was able to drain out during perfusion. Once 400mls of PBS had been infused, a similar volume of PFA was infused through the same catheter.

### **Removal of brain**

Once the perfusion process was completed, the animal was turned over to expose the skull and the skull cleaned. A skin incision was made from the snout to the back of skull and down the neck and spine until mid thorax. Then skin was separated from skull bone, cutting away connective tissue and muscle surrounding the neck and spine. C1 was located and with sharp bone cutters, the bone was cut down either side of the atlas to release the spine. Using fine scissors, the dura matter over spinal column was removed. Starting from the base of the skull, the skull bone was cut in small pieces using bone cutters pulling excised skull away parallel to the surface of the brain. Exposing the cerebellum and working forward, bone was cut and removed over the whole cortex down to the level of the optic socket ensuring no sharp edges were protruding. The dura matter was removed from the surface of brain using fine forceps and fine scissors. Following this the brain, including the cerebellum, was carefully removed from the skull, cutting the olfactory and optic nerves proximally and cutting through any remaining brain stem distally (Figure 2.4 and 2.5).



**Figure 2.4:** Whole brain after post-mortem examination



**Figure 2.5:** Sagittal view of left sided brain

## **Tissue preparation**

The brain, once removed from the skull, was immediately placed in 2% PFA. It was stored at 4°C for 7 days before processing. Once the brain was fixed, coronal slices (5mm thick) of the right hemisphere, starting from anterior to the optic chiasma, were cut, processed and embedded in paraffin wax. Brain tissue was mounted frozen on dry ice, and stored at -80°C until required. The brains were cut on a cryostat and were then sectioned to 5µm thickness onto a slide for subsequent immunohistochemistry staining.

## **Histology**

Qualitative analysis of tissue integrity and neuronal damage was examined in both hemispheres using haematoxylin and eosin (H&E) stain. To assess cell death and glial activation, adjacent sections were stained for nuclear DNA fragmentation using histochemistry with transferase mediated biotinylated d-UTP nick end-labeling (TUNEL), and the appearance of cleaved caspase 3 and microglial ionized calcium-binding adaptor molecule 1 (IBA1) immunoreactivity. For all 3 stains, brain sections were dehydrated in xylene (3x10 min) and rehydrated in graded ethanol solutions (100-70%), followed by double-distilled water.

## **TUNEL staining**

For TUNEL, the sections were pretreated for 15 min in 3% H<sub>2</sub>O<sub>2</sub> in methanol to remove endogenous peroxidase, followed by a 15 min peptidase pre digestion with 20 µg/ml Proteinase K (Promega, UK) at 65°C, and then incubated at 37°C for 2 hours with the TUNEL solution (Roche, UK) containing biotinylated dUTP. The reaction was stopped by incubating the slides for 10 minutes in 300 mM sodium chloride / 300 mM sodium citrate (TUNEL stop solution). The biotin residues were detected with the avidin-biotinylated horseradish peroxidase complex (ABC, Vector Laboratories, Peterborough, UK) and visualized with diaminobenzidine/H<sub>2</sub>O<sub>2</sub> (Sigma, UK), with CoCl<sub>2</sub> and NiCl<sub>2</sub> included to intensify TUNEL histochemistry. The reaction

was stopped in 10mM PB and the sections washed twice in double-distilled water. The sections were dehydrated in 70%, 90%, 95% and 100% ethanol, isopropanol, and twice in xylene, 2 minutes in each before being covered using Depex.

### **Caspase staining**

For Caspase staining, the sections were pretreated for 15 min in 3% H<sub>2</sub>O<sub>2</sub> in methanol to remove endogenous peroxidase activity and processed for antigen retrieval (800 mW microwave irradiation in 0.1M citrate buffer, 10min), followed by overnight incubation with primary rabbit antibody against caspase 3 (1:500). The biotin residues were detected with the avidin-biotinylated horseradish peroxidase complex (ABC, Vector Laboratories, Peterborough, UK) and visualized with diaminobenzidine/H<sub>2</sub>O<sub>2</sub> (Sigma, UK). The sections were counterstained with haematoxylin, dehydrated in graded alcohol and xylene, and mounted with Depex (VWR, UK).

### **Iba1 staining**

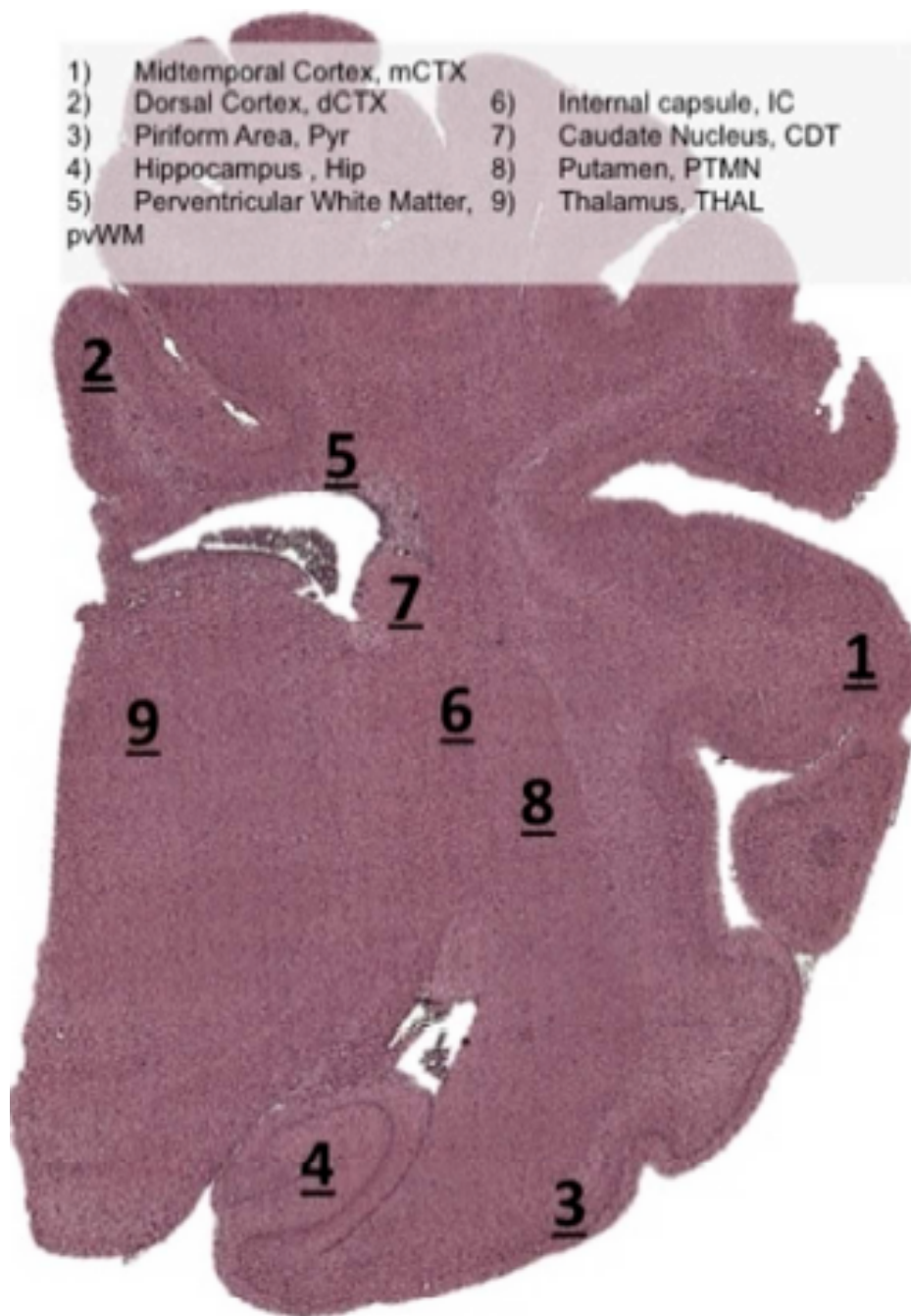
The Iba1 antibody was diluted 1:1000 in Bond Primary Antibody Diluent. The sections were then processed on the Bond Max Automated IHC Stainer, using a pre-programmed staining schedule that dewaxed the slides (using Bond Dewax solution), then pre-treated the sections with Bond Epitope Retrieval solution 2 (an EDTA buffer with surfactant at pH8.8) for 30 minutes. The staining protocol itself used Bond Polymer Refine reagents to block peroxidase/catalase reactivity using 3% Hydrogen Peroxide for 5 minutes, the diluted primary antibody (Ib1a) was then incubated for 15 minutes, followed by a "Post-Primary" incubation, described as a 'polymer penetration enhancer' for 8 minutes, then the Polymer, which was the final layer, for 8 minutes, then with DAB for ten minutes. Bond DAB Enhancer (0.5% Copper sulphate in saline, with surfactant) was then applied for 5 minutes, followed by a Haematoxylin counterstain for 5 minutes. Each step was separated by three brief washes in Bondwash solution (a Tris buffered saline solution, diluted x10 with deionised water

for working solution). The stained sections were then removed and taken to an automated staining machine (Leica ST5020)/cover slipper (Leica CV5030), where the sections were dehydrated through graded alcohols (BDH), cleared in AnalaR xylene (BDH), and mounted with coverslips (BDH) using Pertex Mounting Medium from Cell Path.

### **Quantification of stained cells**

For each animal, quantification was performed in the dorsal cortex (3 regions), mid temporal cortex (insular region), caudate nucleus, thalamus, putamen, periventricular white matter and hippocampus (dentate gyrus). All the counted regions are shown in figure 2.6. The 3 fields were from the superficial, middle and deep cortical layers for cortex and random 3 fields for other regions. TUNEL+ nuclei were counted in 3 fields per region (at 40x magnification, with an area of  $0.76\text{mm}^2$ ) and the average converted into counts per  $\text{mm}^2$ . The less numerous caspase immunoreactive cells were counted at 20x magnification (3 fields,  $3.1\text{mm}^2$ ).

IBA1+ microglial cell bodies and branch density were determined at 40x magnification using a  $0.24 \times 0.24\text{mm}$  square grid, placed in 3 fields for each brain region and counting the number of cell bodies inside the grid (CBD) and the average number of branches (B) crossing the 3 horizontal (top, middle and bottom) and the 3 vertical (left, middle and right)  $0.24\text{mm}$  long gridlines. This determined the microglial ramification index ( $B^2 / \text{CBD}$ ).



**Figure 2.6:** Representative piglet brain photomicrograph indicating brain regions assessed for histology and immunohistochemistry. Permission to reproduce this figure has been granted by Oxford University Press.

## **Magnetic Resonance Spectroscopy (MRS)**

MR spectra were acquired using a 6.5 cm x 5.5 cm elliptical transmit-receive surface-coil tunable for both proton ( $^1\text{H}$ ) and phosphorous ( $^{31}\text{P}$ ) nuclei on a 4.7 Tesla Biospec Avance (Bruker Medizintechnik, Karlsruhe, Germany) (Figure 2.6). Baseline spectra were acquired prior to hypoxia-ischemia, during hypoxia-ischemia and recovery ( $^{31}\text{P}$  only), and thereafter continuously with cycle time  $\sim 5$  h. PRESS  $^1\text{H}$  MR spectra were acquired from 2 positions: ventromedial forebrain centred on both lateral thalami and hypothalami (ventromedial-forebrain; 14mm x 14mm x 7mm voxel); and dorsal right sub-cortical white matter at the centrum semiovale level (WM; 9mm x 4mm x 20mm voxel).  $^1\text{H}$  spectra were acquired with repetition time (TR) 5 sec, 128 summed transients, and echo times (TE) 25 ms, 144 ms, and 288 ms.  $^{31}\text{P}$  MR spectra were acquired from whole brain using single-pulse acquire with TR 10 s.

## **MRS Analysis**

$^1\text{H}$  spectra were analysed using LCModel. For each spectrum the relative signal amplitudes of choline (Cho), total creatine (Cr), N-acetyl-aspartate (NAA) and lactate (Lac) were measured. For statistical analysis, the primary  $^1\text{H}$  MRS outcome measure nominated a priori was Lac/NAA. Metabolite peak-area ratios were plotted from baseline to 48 h after hypoxia-ischemia for each voxel in each subject and analysed according to the randomized group.



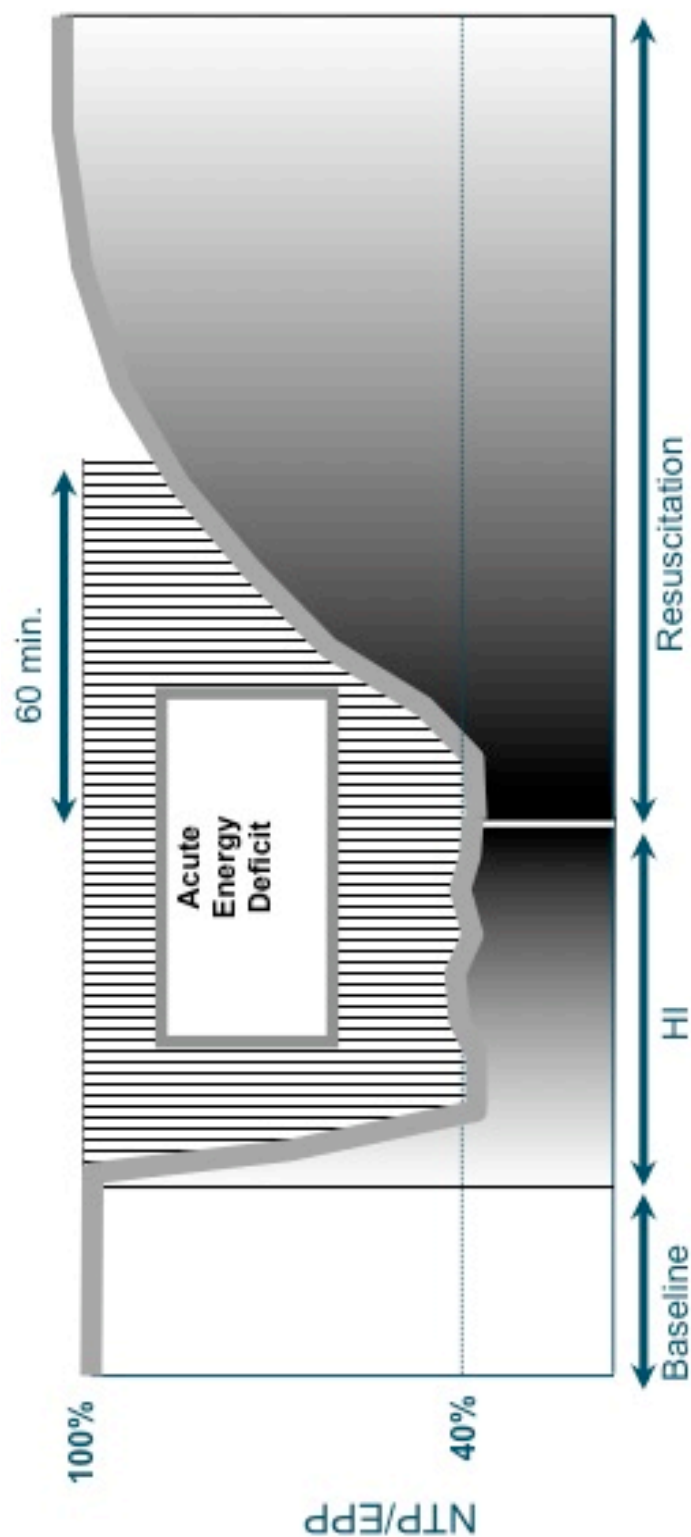
**Figure 2.7:** 4.7 Tesla Bruker MR system in the designated magnet room



$^{31}\text{P}$  MR spectra were analysed by AMARES. Signal amplitudes and chemical shifts were measured for phosphocreatine (PCr), Pi (3 independent components fitted) and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -NTP; the equivalent signal amplitude for EPP was also calculated. Intracellular pH ( $\text{pH}_i$ ) was measured from the difference between the PCr and amplitude-weighted mean Pi chemical shifts. Metabolite peak-area ratios were plotted from baseline to 48 h after hypoxia-ischemia for each experimental group.

### **Cerebral hypoxia-ischemia**

Whilst in the MRS system, transient hypoxia-ischemia was induced by remote occlusion of both the common carotid arteries, using the inflatable vascular occluders, and reducing fractional inspired ( $F_i$ )  $\text{O}_2$  to 12 % (v/v). During transient hypoxia-ischemia cerebral-energetic changes were observed every 2 min by phosphorus ( $^{31}\text{P}$ ) MRS and the  $\beta$ -nucleotide triphosphate (NTP; mainly ATP) peak height was automatically generated. Hypoxia-ischemia was continued until 10 min after the  $\beta$ -NTP peak height had fallen to 40% of baseline for Xenon experiment and 30% of baseline for Amiloride experiment. During this 10 min period,  $F_i\text{O}_2$  was adjusted in order to keep  $\beta$ -NTP peak height as close to the expected of baseline as possible. At the end of this 10 min period, the occluders were deflated and  $F_i\text{O}_2$  was normalised.  $^{31}\text{P}$  MR spectra were acquired for a further hour to monitor recovery from hypoxia-ischemia. The time integral of the change in  $\beta$ -NTP peak area relative to the exchangeable phosphate pool ( $\text{EPP} = \text{inorganic phosphate (Pi)} + \text{phosphocreatine (PCr)} + (2\gamma + \beta)\text{-NTP}$ ) during hypoxia-ischemia and the first 60 min of resuscitation gave the magnitude of acute energy depletion (AED).



**Figure 2.8:** Quantification of insult severity. Global hypoxic and cerebral ischemic insult by occlusion of both carotid arteries and reduction of oxygen until  $\beta$ -NTP 40% baseline, held for 10 minutes in Xenon study

## **Experimental groups**

In this thesis, I am discussing the data of two of our experimental series investigating neuroprotective therapies. My primary involvement in these studies includes all histological methods (staining and quantification) and correlating MRS biomarkers with these histological markers using appropriate statistical methods. I also contributed towards data collection and the studies for both the experiments. A team of physicists and scientists helped with the experiments. However, I have worked along them in this model using the Bruker 4.7T system (approximately 30 experiments) as well as in a further two experimental series on the Varian 9.4T MR system. These studies have given me insight and knowledge about the animal model and the science behind the animal research. All the data analysed and discussed in the following chapters are from the first 2 neuroprotection experiments on the Bruker MR system.

### **1. Xenon/ Hypothermia experiment**

Baseline data were acquired before transient hypoxia-ischemia but after stabilization of the animal in the MRS system. Following transient hypoxia-ischemia, 36 piglets were randomised into 4 groups (each  $n=9$ ), with intervention from 2-26 h: Group (i) normothermia ( $38.5^{\circ}\text{C}$ ); Group (ii) normothermia + 24 h 50% inhaled xenon; Group (iii) 24 h therapeutic hypothermia (rectal temperature ( $T_{\text{rectal}}$ )  $33.5^{\circ}\text{C}$ ) or Group (iv) 24 h 50% inhaled xenon +24 h therapeutic hypothermia ( $T_{\text{rectal}}$   $33.5^{\circ}\text{C}$ ). Normothermic piglets were maintained at their target  $T_{\text{rectal}}$  using a warmed water mattress under the piglet and circulating warm air; hypothermic piglets (Groups (iii) and (iv)) were cooled (by water mattress) over 90-120 min to a target  $T_{\text{rectal}}$  of  $33.5^{\circ}\text{C}$ , which was maintained between 2-26 h following hypoxia-ischemia. At 26 h after hypoxia-ischemia piglets were re-warmed to normothermia at

0.5°C/h (Faulkner, Bainbridge et al. 2011).

## **2. Methyl Isobutyl Amiloride (MIA) experiment**

Baseline data were acquired before transient hypoxia-ischemia but after stabilization of the animal in the MRS system. Following transient hypoxia-ischemia, 18 male piglets (<24 h of age) were randomized to 2 groups (each n=9) (1) normothermia; or (2) 2.5mg/kg of MIA at 10 mins after resuscitation and 8 hourly thereafter (Robertson, Kato et al. 2013).

## **Statistics**

All metabolite ratios were converted to a logarithmic (log) scale before analysis. The integrating, 48h Area Under the Curve (AUC) data for the metabolite ratios were calculated from the log values. Where metabolite ratios decreased during the 48h after HI (PCr/ePP, NTP/ePP, NAA/Cr), the ratio-specific integral value was determined using Area Over the Curve (AOC); AOC was also used for Acute Energy Deficit (AED) and for assessing pH changes. Area under the curve (AUC) was used as a generic term for the whole set of MRS outcome measures. When a specific metabolite is mentioned, it is AUC in case of metabolite ratios increasing after HI (Lac/Cr, Lac/NAA, Pi/ePP), and AOC in case of decreasing ones. All statistics for histopathology outcomes were performed on data transformed by the  $f=\log(x+a)$  algorithm with  $a=1$  for TUNEL and caspase 3+ density, and  $a=0.1$  for microglial ramification. All other ratios (metabolite ratios, actual/expected ratios) were analysed using simple log values.

Predictive Analytics Software 18 (PASW; IBM, USA) was used for all statistical for late biomarkers analysis. Multiple linear regression analysis was done to assess the relationship of early and late biomarkers with the outcome. Statistical significance of the  $R^2$  value was assessed using the F-test. The differences between the groups detected using one-way ANOVA, followed by post-hoc Tukey test. Paired and

unpaired t test was used when appropriate. Correlation plot  $R^2$  values were tested for significance using F-test.

## RESULTS

# **Chapter 3 - The Predictive value of Early MRS Biomarkers (acquired within 4 hours after HI) without neuroprotective intervention in a piglet model of HIE**

## **Introduction**

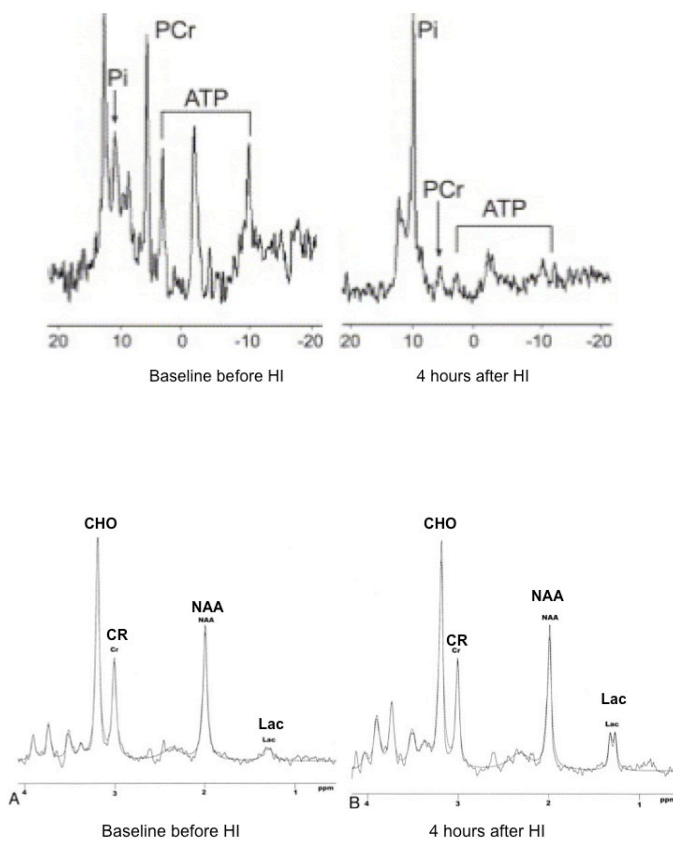
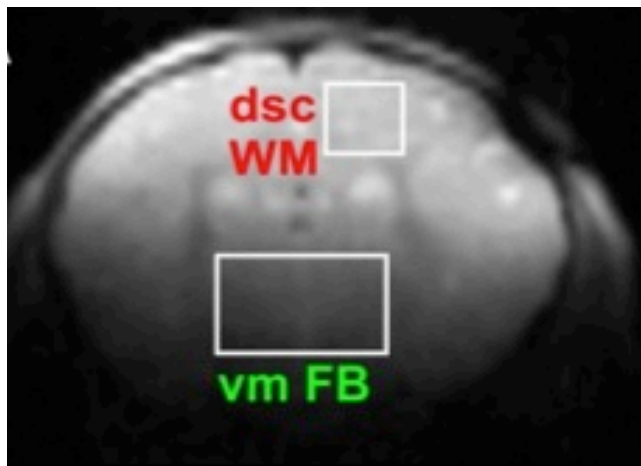
The course of the encephalopathy following hypoxia-ischemic (HI) episodes is very variable and it is difficult to predict the outcome of an individual patient early and particularly early after reperfusion. A better prediction of the outcome at early time points would greatly facilitate future neuroprotection trials and clinical decisions. In order to accelerate translation of research to clinical practice, biomarkers are needed to better quantify the severity of the hypoxic-ischemic brain injury. This will help us to select the right candidate, who would develop moderate or severe encephalopathy, for future neuroprotection trials.

We defined MRS data (proton and phosphorus) that were acquired at first time point after the HI as early biomarkers (EBM). Our aim was to investigate early MRS biomarkers (EBM), acquired between 2 and 4 h after HI, and their correlation to MRS and histological outcome in a piglet model of HIE.

## **Materials and methods**

Experiments were carried out according to United Kingdom Home Office guidelines. Large-white male piglets, aged < 24h were anaesthetised and surgically prepared as described previously (Lorek, Takei et al. 1994, Faulkner, Bainbridge et al. 2011). All physiological monitoring and procedures during experiment were done as explained previously (Faulkner, Bainbridge et al. 2011). All data used in the current study were from two large translational neuroprotection studies in a piglet model of hypoxic-ischemia. The first study (Faulkner, Bainbridge et al. 2011) explored the combination of hypothermia and Xenon (Xe/HT), the second (Robertson, Kato et al. 2013) the treatment with Methyl-Isobutyl-Amiloride (MIA). MRS acquisition (Figure 3.1) and analysis, statistics, histological staining and quantification and hypoxia-ischemia were as described in previous chapter.





**Figure 3.1:** Voxel position, typical proton and phosphorus spectra; baseline and 4 hours after HI in untreated animals.

## Physiological Measures

There were no intergroup differences in body weight, postnatal age, baseline physiological (HR and MABP) and biochemical measures (blood lactate, base excess and glucose) (**Appendix Tables 1 and 2**). Insult severity qualified with triphosphate levels and PaO<sub>2</sub> and PaCO<sub>2</sub> were similar between groups during HI and time to reach target rectal temperature during post HI hypothermic induction was similar.

During cooling induction (2-3.5h), maintenance (3.5-26h) and re-warming/normothermia (26-48h) periods, mean HR and MABP and blood chemistry and electrolytes (pH, lactate, base excess, glucose) were similar between groups (**Appendix Tables 1 and 2**). In the MIA study, two piglets from the saline placebo group died at 24 and 32 h; 1 MIA piglet died at 30h because of complications secondary to severe HI. All other subjects survived until the end of experiment, 48 hours after the insult. In the Xe/HT study, four piglets were terminated prior to 48-hour post insult due to ventilator problems in 2 cases and complications secondary to severe hypoxia–ischemia (1 case each from the xenon alone and hypothermia alone groups and 2 cases from the combined xenon and hypothermia group).

## Description of Early Biomarkers and Outcome data

For early biomarkers, we used the single point MRS data from the first time point, which is acquired 2-4 h after completion of HI insults (Tables 3.1 and 3.2). These data include <sup>1</sup>H MRS WM and thalamic voxels (Lac/Cr, NAA/Cr and Lac/NAA), and the <sup>31</sup>P MRS whole brain voxel (Pi/ePP, PCr/ePP, NTP/ePP and pH(Pi)). We also used Acute Energy Deficit (AED), which was calculated as described previously. All these 11 markers are described as early biomarkers (EBMs).

Group	<sup>31</sup> P-Whole Brain time in hours mean±SEM (n)	<sup>1</sup> H-WM voxel time in hours mean±SEM (n)	<sup>1</sup> H-thalamic voxel time in hours mean±SEM (n)
Control	2.04±0.02 (8)	3.95±0.09 (9)	2.97±0.04 (9)
Xenon	2.02±0.01 (8)	4.39±0.17 (9)	3.40±0.17 (9)
Hypothermia	2.11±0.05 (7)	4.43±0.17 (9)	3.34±0.12 (9)
Combined	1.99±0.04 (8)	3.96±0.13 (8)	3.09±0.11 (9)

**Table 3.1:** Early Marker Acquisition Time Point (hours) and Group Size for Xe/HT study

Group	<sup>31</sup> P-Whole Brain time in hours mean±SEM (n)	<sup>1</sup> H-WM voxel time in hours mean±SEM (n)	<sup>1</sup> H-thalamic voxel time in hours mean±SEM (n)
Saline	4.47±0.24 (9)	3.52±0.21 (9)	2.56±0.19 (9)
MIA	5.11±0.39 (9)	3.80±0.16 (9)	2.71±0.13 (9)

**Table 3.2:** Early Marker Acquisition Time Point (hours) and Group Size for MIA study

We compared EBMs with MRS area under the curve data (a value from all the MRS data points over 48h to give a value encompassing information about the trajectory of injury) and histological and immunohistochemistry data. MRS data included twelve outcomes (area under the curve); 4 from the  $^1\text{H}$  dorsoparietal subcortical white matter (WM) voxel (Lac/Cr, Cho/Cr, NAA/Cr and Lac/NAA), 4 from the  $^1\text{H}$  ventromedial forebrain (thalamic) voxel (Lac/Cr, Cho/Cr, NAA/Cr and Lac/NAA), and 4 from the  $^{31}\text{P}$  whole brain voxel (Pi/ePP, PCr/ePP, NTP/ePP and pH(Pi)). Neither the WM nor thalamic Cho/Cr AUC data showed insult-specific change in either of the two studies, and thus not included in further analysis. For histopathology outcomes, density of TUNEL+ nuclei, density of cleaved caspase 3 positive cells, and the ramification index were used. In further analysis, individual histopathology outcomes for each animal and staining were combined by using an average across all regions, with the intention to reduce the number of outcomes to 3 for each animal. Previous analysis revealed that these averaged histopathology values exhibited considerably higher correlation with the biomarker AUCs than those observed for region-specific histopathology (Faulkner et al, 2011). Overall, a total of 13 standardized outcomes, from MRS and histology, were used for analysis.

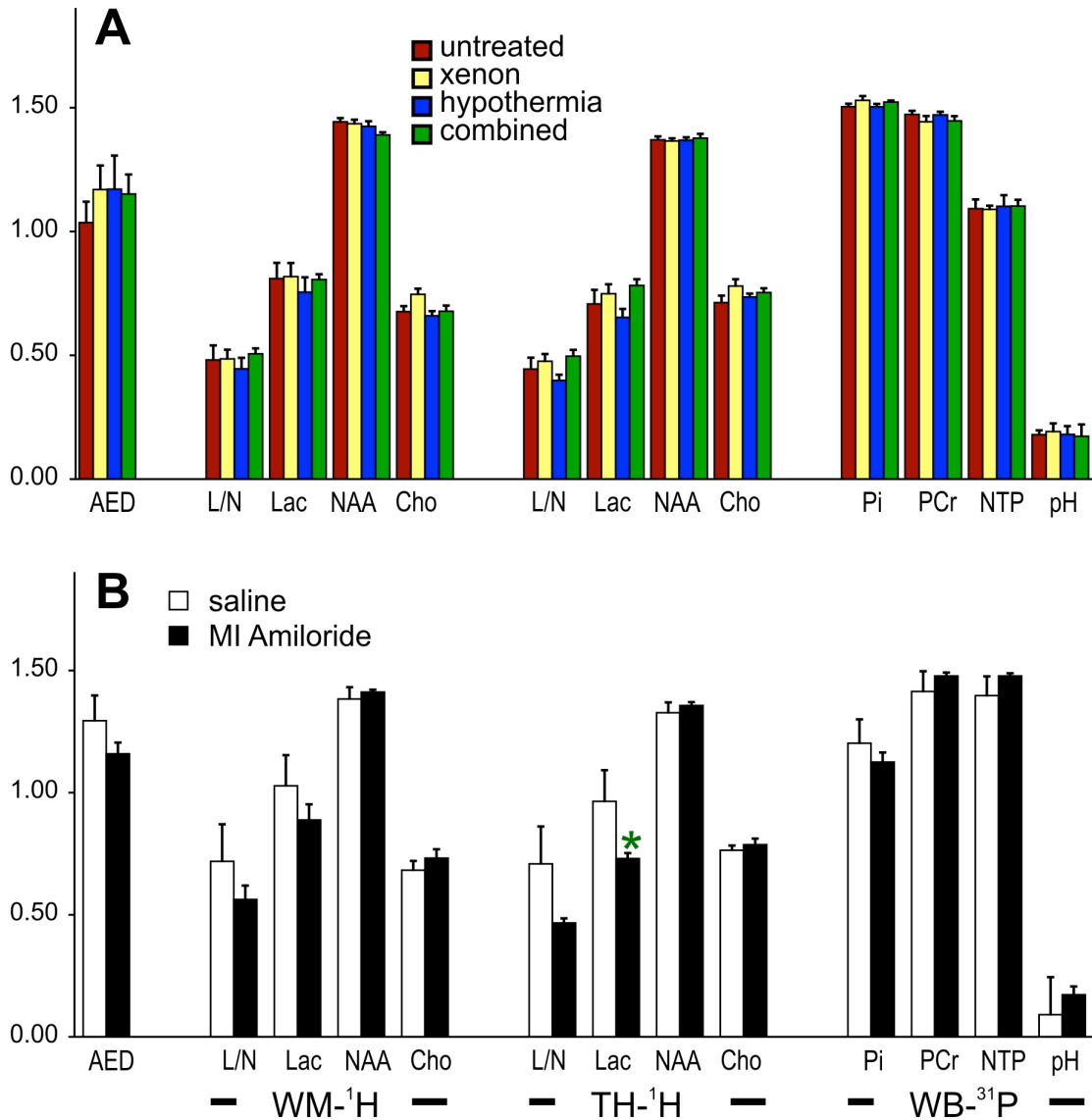
## Results

In each animal, EBMs for  $^{31}\text{P}$  whole brain,  $^1\text{H}$  WM voxel, and  $^1\text{H}$  thalamic voxel were acquired consecutively:  $^{31}\text{P}$  MRS-derived EBMs were obtained between 1.7-2.3 hours after HI insult, those for the  $^1\text{H}$  thalamic voxel at 2.8-4.1 hours, and for the  $^1\text{H}$  WM at 3.6-5.5 hours in Xe/HT study. The mean and standard error of the mean for the acquisition time point for Xe/HT and MIA studies, and the number of animals with this first data point inside the above time frames are shown in tables 3.1 and 3.2. There was no difference in the acquisition time point for all EBMs between the subgroups in both studies.

The values of all early biomarkers peak area ratios of all control animals are given in the table 3.3 and figure 3.2. The mean for all increasing biomarkers for Xe/HT study are generally lower than those in MIA study, appropriately indicating the different severity in both studies (table 3.3). There is no significant difference in the mean value of all EBMs among all the study groups in Xe/HT study (figure 3.2A). However, compared with the saline controls shown in figure 3.2B, the average “increasing” EBM values (Lac/Cr, Lac/NAA, Pi/ePP) for the MIA group were consistently lower, and “decreasing” EBMs (NAA/Cr, PCr/ePP, NTP/ePP) higher, reaching statistical significance in the case of thalamic Lac/Cr ( $p < 0.05$ ). This may be due to the treatment effect as MIA is administered 10 minutes after HI in the treatment group.

<b>Early Biomarkers</b>	<b>Xe/HT study Mean (SD)</b>	<b>MIA study Mean (SD)</b>
Thalamic Lac/NAA	0.41 (0.11)	0.72 (0.48)
Thalamic Lac/Cr	0.67 (0.14)	0.97 (0.40)
Thalamic NAA/Cr	1.37 (0.04)	1.32 (0.13)
WM Lac/NAA	0.46 (0.18)	0.72 (0.48)
WM Lac/Cr	0.79 (0.19)	1.03 (0.40)
WM NAA/Cr	1.44 (0.04)	1.38 (0.15)
AED	0.10 (0.02)	0.13 (0.03)
Pi/EPP	0.21 (0.04)	0.22 (0.05)
PCr/EPP	0.28 (0.03)	0.27 (0.09)
NTP/EPP	0.29 (0.04)	0.27 (0.08)
pH (Pi)	7.17 (0.04)	7.07 (0.49)

**Table 3.3:** MRS Early Biomarkers (EBMs) peak area ratios acquired between 2 and 4 hours after HI for the Xe/HT and MIA study. Data shown as Mean and standard deviation



**Figure 3.2:** MRS Early Biomarker (EBMs) peak area ratios acquired between 2 and 4 hours after HI for the Xe/HT (A) and MIA study (B). All bars show mean $\pm$ standard error of the mean (SEM),  $n = 9$  animals per subgroup. The Y-axis is a logarithmic scale of the peak area ratio. The Xe/HT study consisted of 4 subgroups – control (ctrl), Xenon-treated (Xe), hypothermia (HT) and combined hypothermia and Xenon (Xe/HT). The MIA study consisted of only two groups – controls treated with saline and methyl-isobutyl amiloride (MI Amiloride). \* $p < 0.05$  in t-test for difference between saline and MIA-treated subgroups. AED – Acute Energy Deficit, Cho – Choline to creatine ratio, L/N – Lactate to N-Acetyl Aspartate (NAA) ratio, Lac – Lactate to creatine ratio, NAA – NAA to creatinine, NTP – Nucleotide triphosphate to exchangeable phosphate pool, PCr – phosphocreatine to exchangeable phosphate pool, Pi – inorganic phosphorus to exchangeable phosphate pool, TH – thalamic, WB – Whole Brain, WM – dorsal subcortical White Matter.

### **Correlation of EBMs with MRS and Histopathology Outcomes in the Xe/HT study**

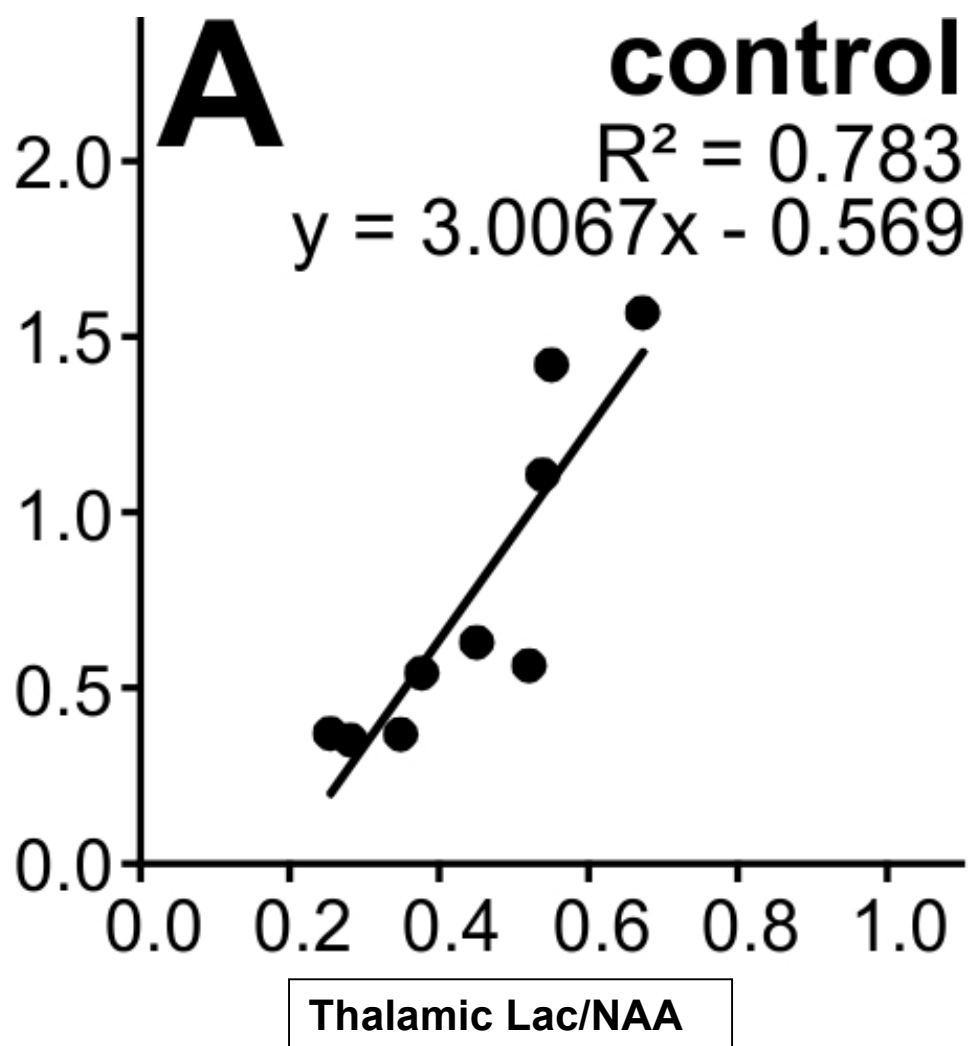
On regression analysis, thalamic Lac/NAA EBM showed positive and significant correlation with the thalamic Lac/NAA AUC outcome as shown in figure 3.3 ( $p < 0.05$ ) and table 3.4. On analysing with all remaining 12 outcomes for the untreated, control group, thalamic Lac/NAA showed a strong and significant correlation with  $R^2$  values (Figure 3.4). The only exception was the correlation with the log caspase 3 density (Figure 3.4F), where  $R^2$  value was low (0.11) and did not reach statistical significance ( $p = 0.53$ ). Of the 12 significant correlations, 11 displayed a positive regression slope and ramification index (Figure 3.4L) a negative slope.

The results of regression analysis of all EBMs with outcome data are summarized in Table 3.4. On univariate regression analysis, as shown in table 3.4, the  $R^2$  values were particularly high for thalamic Lac/NAA EBM and moderate for thalamic Lac/Cr, WM Lac/Cr, and WM Lac/NAA. In the Xe/HT study, EBMs belonging to the “decreasing” group - WM and thalamic NAA/Cr, PCr/ePP, NTP/ePP – all displayed low  $R^2$  values.

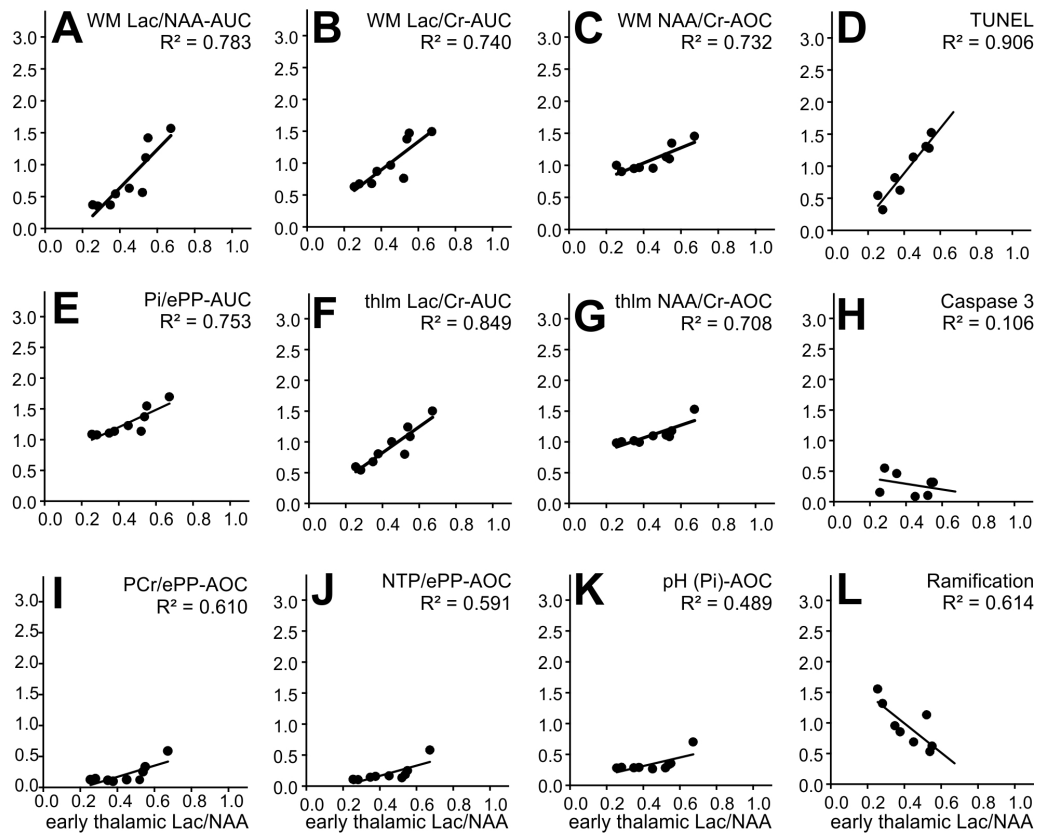


Outcome	wm Lac/NAA		wm Lac/Cr		wm NAA/Cr		thLac/NAA		thLac/Cr		th NAA/Cr		PCr/EPP		NTP/EPP		PI/EPP		pH(PI)		TUNEL		RAM	
	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p
Early Biomarkers																								
AED	0.34	0.13	0.32	0.14	0.37	0.11	0.29	0.17	0.31	0.15	0.28	0.18	0.19	0.28	0.16	0.33	0.28	0.18	0.13	0.39	0.50	0.05	0.26	0.20
PI/EPP	0.38	0.10	0.39	0.10	0.34	0.13	0.34	0.13	0.43	0.08	0.25	0.20	0.15	0.35	0.23	0.22	0.35	0.13	0.10	0.44	0.58	0.03	0.54	0.04
wmLac/Cr	<b>0.81</b>	<b>&lt;0.01</b>	<b>0.89</b>	<b>&lt;0.01</b>	<b>0.53</b>	<b>0.04</b>	<b>0.48</b>	<b>0.06</b>	<b>0.60</b>	<b>0.02</b>	<b>0.26</b>	<b>0.20</b>	<b>0.45</b>	<b>0.07</b>	<b>0.26</b>	<b>0.20</b>	<b>0.69</b>	<b>0.01</b>	<b>0.21</b>	<b>0.26</b>	<b>0.46</b>	<b>0.06</b>	<b>0.52</b>	<b>0.04</b>
wmLac/NAA	<b>0.79</b>	<b>&lt;0.01</b>	<b>0.84</b>	<b>&lt;0.01</b>	<b>0.58</b>	<b>0.03</b>	<b>0.42</b>	<b>0.08</b>	<b>0.52</b>	<b>0.04</b>	<b>0.25</b>	<b>0.21</b>	<b>0.43</b>	<b>0.08</b>	<b>0.23</b>	<b>0.23</b>	<b>0.67</b>	<b>0.01</b>	<b>0.18</b>	<b>0.30</b>	<b>0.50</b>	<b>0.05</b>	<b>0.44</b>	<b>0.07</b>
thLac/Cr	<b>0.69</b>	<b>0.01</b>	<b>0.62</b>	<b>0.02</b>	<b>0.73</b>	<b>0.01</b>	<b>0.74</b>	<b>0.01</b>	<b>0.75</b>	<b>0.01</b>	<b>0.67</b>	<b>0.01</b>	<b>0.53</b>	<b>0.04</b>	<b>0.55</b>	<b>0.04</b>	<b>0.65</b>	<b>0.02</b>	<b>0.45</b>	<b>0.07</b>	<b>0.86</b>	<b>&lt;0.01</b>	<b>0.46</b>	<b>0.07</b>
thLac/NAA	<b>0.78</b>	<b>&lt;0.01</b>	<b>0.74</b>	<b>0.01</b>	<b>0.73</b>	<b>0.01</b>	<b>0.82</b>	<b>&lt;0.01</b>	<b>0.85</b>	<b>&lt;0.01</b>	<b>0.71</b>	<b>0.01</b>	<b>0.61</b>	<b>0.02</b>	<b>0.59</b>	<b>0.03</b>	<b>0.75</b>	<b>0.01</b>	<b>0.49</b>	<b>0.05</b>	<b>0.91</b>	<b>&lt;0.01</b>	<b>0.61</b>	<b>0.02</b>
wmNAA/Cr	0.37	0.11	0.29	0.17	0.50	0.05	0.12	0.39	0.12	0.40	0.15	0.35	0.17	0.31	0.08	0.50	0.29	0.17	0.05	0.58	0.38	0.10	0.05	0.59
thNAA/Cr	0.14	0.35	0.25	0.21	0.01	0.88	0.11	0.42	0.17	0.31	0.03	0.68	0.08	0.50	0.03	0.69	0.15	0.34	0.01	0.78	0.08	0.51	0.41	0.08
pH/PI	0.28	0.17	0.28	0.18	0.18	0.29	0.36	0.12	0.30	0.16	0.39	0.10	0.33	0.14	0.27	0.19	0.32	0.15	0.26	0.19	0.13	0.37	0.20	0.26
PCr/EPP	0.19	0.28	0.19	0.27	0.08	0.50	0.13	0.39	0.08	0.49	0.14	0.35	0.30	0.16	0.19	0.28	0.23	0.23	0.23	0.23	0.04	0.63	0.03	0.68
NTP/EPP	0.07	0.54	0.06	0.55	0.02	0.77	0.06	0.57	0.02	0.72	0.07	0.53	0.22	0.24	0.12	0.41	0.10	0.46	0.19	0.27	0.22	0.24	0.02	0.76

**Table 3.4:** Correlation between EBM<sub>s</sub> and the entire 12 outcome measures in Xe/HT study, The R<sup>2</sup> and p values are obtained by univariate linear regression. EBM<sub>s</sub>, significant highly and with most outcome measures are bold. Thalamic Lac/Naa is highlighted with blue, as this was the only EBM, which significantly correlated with all the outcome measures



**Figure 3.3:** Thalamic Lac/NAA EBM (X-Axis) shows significant correlation with post-HI thalamic Lac/NAA Area under the curve (AUC) (Y-Axis) in the control group of the Xe/HT study. The formulas represent regression line functions.



**Figure 3.4:** Correlation of thalamic Lac/NAA EBM (X-Axis) with in 9 additional MRS and 3 histopathology outcomes (Y-Axis) in the control subgroup of the Xe/HT study. Note the consistently high correlation  $R^2$  values ( $p < 0.05$ ) with MRS AUCs for WM Lac/NAA (A), Lac/Cr (B) and NAA/Cr (C), thalamic Lac/Cr (F) and NAA/Cr (G), whole brain Pi/ePP (E), PCr/EPP (I), NTP/ePP (J) and pH (K), as well as with histopathology values for log TUNEL density (D) and log microglial ramification (L). Correlation plot against log caspase 3 density (H) was the only exception, with an  $R^2$  value of 0.106 ( $p = 0.33$ ).

## **Correlation of EBMs with MRS and Histopathology Outcomes in the MIA study**

To revalidate the predictive value of EBMs, the same analysis protocols were used for data from the MIA treatment study in a more severe form of HI insult. All 11 markers (5 increasing EBMs, 4 decreasing EBMs, pH and AED) showed higher correlation for MRS and histopathology outcomes than those observed in the Xe/HT study (Table 3.5) with statistical significance ( $p < 0.01$ ). Correlation of thalamic Lac/NAA EBM was similarly high and significant with all MRS and histological outcome measures. All biomarkers, from both increasing and decreasing group, correlated with the outcome measure significantly (table 3.5)

## **Correlation with Multivariable Regression**

As all the EBMs showed good correlation with the outcome measures by univariate regression model, we further analysed the data using multivariable regression model. We used Tunel density as the outcome measure for this analysis and all EBMS were entered as predictive variables. STEPWISE method of multivariable regression was used. Thalamic Lac/NAA showed high and significant correlation with Tunel density ( $p = < 0.001$ ) when compared to other EBMS, as shown in Table 3.6. This was true for both Xe/HT and MIA studies.

Outcome	wm Lac/NAA		wm Lac/Cr		wm NAA/Cr		thLac/NAA		thLac/Cr		th NAA/Cr		PCr/EPP		NTP/EPP		PI/EPP		pH(Pi)		TUNEL		RAM	
	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p
Early Biomarkers																								
AED	0.36	0.12	0.34	0.13	0.36	0.12	0.48	0.06	0.31	0.15	0.59	0.03	0.26	0.20	0.43	<0.01	0.35	0.12	0.41	0.09	0.24	0.22	0.01	0.94
PI/EPP	0.75	0.01	0.71	0.01	0.77	<0.01	0.84	<0.01	0.75	0.01	0.87	<0.01	0.72	0.01	0.80	<0.01	0.74	0.01	0.80	<0.01	0.51	0.05	0.38	0.10
wmLac/Cr	0.92	<0.01	0.93	<0.01	0.85	<0.01	0.83	<0.01	0.89	<0.01	0.78	<0.01	0.91	<0.01	0.90	<0.01	0.92	<0.01	0.71	0.01	0.71	0.01	0.37	0.11
wmLac/NAA	0.98	<0.01	0.96	<0.01	0.96	<0.01	0.95	<0.01	0.95	<0.01	0.92	<0.01	0.92	<0.01	0.97	<0.01	0.94	<0.01	0.89	<0.01	0.76	0.01	0.76	<0.01
thLac/Cr	0.84	<0.01	0.83	<0.01	0.80	<0.01	0.91	<0.01	0.86	<0.01	0.91	<0.01	0.75	0.01	0.83	<0.01	0.82	<0.01	0.86	<0.01	0.43	0.08	0.40	0.09
thLac/NAA	0.87	<0.01	0.85	<0.01	0.84	<0.01	0.96	<0.01	0.89	<0.01	0.97	<0.01	0.77	<0.01	0.84	0.04	0.80	<0.01	0.95	<0.01	0.53	0.04	0.49	0.05
wmNAA/Cr	0.38	0.10	0.31	0.15	0.48	0.06	0.54	0.04	0.32	0.14	0.68	0.01	0.27	0.18	0.53	0.01	0.41	0.09	0.53	0.04	0.13	0.38	0.25	0.20
thNAA/Cr	0.61	0.02	0.55	0.03	0.66	0.01	0.76	<0.01	0.55	0.04	0.87	<0.01	0.51	0.05	0.67	0.02	0.56	0.03	0.74	0.01	0.39	0.10	0.20	0.27
pH/Pi	0.44	0.07	0.42	0.08	0.46	0.06	0.50	0.05	0.35	0.12	0.60	0.02	0.36	0.11	0.61	0.01	0.55	0.04	0.41	0.09	0.23	0.23	0.09	0.47
PCr/EPP	0.65	0.02	0.68	0.01	0.58	0.03	0.75	0.01	0.69	0.01	0.75	0.01	0.60	0.02	0.71	0.01	0.72	0.01	0.70	0.01	0.50	0.05	0.13	0.38
NTP/EPP	0.59	0.03	0.50	0.05	0.71	0.01	0.67	0.01	0.52	0.04	0.74	0.01	0.53	0.04	0.70	0.01	0.58	0.03	0.57	0.03	0.29	0.17	0.26	0.20

**Table 3.5:** Correlation between EBM and the entire 12 outcome measures in MIA study, The R<sup>2</sup> and p values are obtained by univariate linear regression. Thalamic Lac/Naa is highlighted with blue, as this was the only EBM, which significantly correlated with all the outcome measures

Model	Study	Variable	Adjusted R Square	Standard error of estimate	F change	p value
Stepwise	Xe/HT	thLac/NAA	0.89	0.14	59.2	<0.001
	MIA	thLac/NAA	0.73	0.23	38.1	<0.001

**Table 3.6:** Results of multivariable regression model using Tunel density as outcome measure and all the EBMs as predictive variables.

## Discussion

We have shown that, by measuring EBMs between 2 and 4 h after hypoxic-ischemic insult, MRS predicts the MRS and histo-pathological outcome at 48 hours in control animals. Out of all EBMs, thalamic Lac/NAA showed a high significant correlation with all possible outcome measures in both studies. Previous studies in neonatal piglets showed that 5 of these EBMs (WM and thalamic Lac/Cr and Lac/NAA, and Pi/ePP) increased during the 48 hours after HI insult. Four of the biomarkers (WM and thalamic NAA/Cr, PCr/ePP, NTP/ePP) decreased, and pH(Pi) first showed a moderate early increase (alkalotic shift) by 0.05-0.1 pH points, followed by a precipitate drop in case of secondary energy failure (Robertson et al., 2011). In our study, EBMs belonging to the “increasing” group all showed statistically significant increase with most outcome parameters (Tunel density, Lac/NAA, Lac/Cr, Pi/EPP), a significant decrease with microglial ramification and absence of significant correlation for caspase 3.

The course of cerebral Lac after hypoxic-ischemic insult has been well demonstrated in experimental studies. Cerebral Lac increases immediately after hypoxia-ischemia, and almost normalizes within 30 minutes of starting reperfusion (Lei H and J 1998), after which there is a secondary, delayed Lac rise after several hours (Penrice,

Lorek et al. 1997). The immediate Lac production following hypoxia-ischemia is due to anaerobic glycolysis in astrocytes stimulated by glutamate excitotoxicity (Pellerin, Bouzier-Sore et al. 2007). In addition it is well known that NAA is a neuronal marker, and its fall correlates with neuronal death (Sager, Hansen et al. 2000). However, it has been shown that fall in NAA after focal ischemia occurs more gradually than increase in Lac (Barker P 2005). NAA is synthesized in neuronal mitochondria using L-aspartate and acetyl coenzyme A through the reaction of L-aspartateN-acetyltransferase enzyme. It has been shown that synthesis of NAA as a marker of the integrity of neuronal mitochondrial metabolism (Moffett, Ross et al. 2007). There is a direct relationship between NAA synthesis, oxygen consumption, and ATP production by the mitochondria. Thus high correlation of thalamic Lac/NAA EBM with outcome observed in our study may be due to Lac increase rather than fall in NAA. However significant correlation of both thalamic and WM NAA/Cr with outcome in MIA study (more severe insult), suggests that NAA could start to decrease early if there was a severe hypoxia-ischemia.

When ATP generation is impaired, energy flux is maintained by the creatine kinase equilibrium with the breakdown of PCr and increase in Pi, so that a decline in PCr and increase in Pi are valuable indicators of impaired energy metabolism even in the presence of normal or near normal NTP (mainly NTP). Therefore PCr and Pi gives a non-invasive measure of the adequacy of cerebral oxidative metabolism. Several studies demonstrated that phosphorus ( $^{31}\text{P}$ ) MRS frequently shows apparently normal brain energy metabolism soon after hypoxia-ischemia (Hope, Costello et al. 1984). However, for infants with eventual adverse outcome, during the first days of life PCr and NTP subsequently decline, and Pi increases despite adequate resuscitation (Azzopardi, Wyatt et al. 1989, Cady, Amess et al. 1997). This was termed as secondary energy failure. The severity of secondary energy failure was related to the pathology of hypoxia-ischemia, thus related to outcome in clinical and

preclinical studies. Raised Pi/EPP, acquired at 2 h after hypoxia-ischemia, correlated with secondary energy failure in our piglet model (Cady, Iwata et al. 2008). Of the <sup>31</sup>P-MRS EBMs, our study revealed that Pi/EPP had the highest correlation with outcome, followed by PCr/EPP, and then by NTP/EPP. Pi/EPP had a significant correlation with outcome in all forms of injury, but PCr/EPP and NTP/EPP showed significant correlation only in severe injury.

One of the important characteristic to be a valid biomarker is to have a strong biological basis, which is in the same pathological pathway as the clinical outcome. Neuronal cell death following hypoxia-ischemia could be apoptotic or necrotic. The most extensively used marker for cell death is based on the integrity of nuclear DNA (Wyllie 1980), as DNA degradation indicates irreversible stage of cell death. This DNA fragmentation is identified easily and completely by the terminal transferase-mediated dUTP nick-end labelling (TUNEL) method (Gavrieli, Sherman et al. 1992). In addition, activation of microglia has been shown in animal models of excitotoxic and hypoxic-ischemic brain injury (Hagberg, Peebles et al. 2002). When activated, microglia can take a deramified amoeboid form, thus ramification index could help in quantifying the severity of hypoxia-ischemia (Faulkner et al 2011). We observed strong and significant correlation of Pi/EPP, thalamic and WM Lac/NAA and Lac/Cr with TUNEL detected cell death and microglial ramification index. This demonstrates the strong biological association of these EBMs.

As demonstrated in the current meta-analysis based on therapeutic studies for combination of Xenon and hypothermia (Faulkner, Bainbridge et al. 2011), and for methyl-isobutyl Amiloride, in neonatal piglets (Robertson et al., submitted), EBMs acquired with MRS within 2-4h after insult showed a significant correlation with the later MRS and histopathology outcome. The extent of correlation was dependent on marker, region and insult severity. Increasing EBMs that increased after HI insult



(Lac/Cr, Lac/NAA and to some extent Pi/ePP) revealed significant correlation with outcome in the control group in the mild to moderate severity insult (Xe/HT). With a more severe insult in the MIA study, significant correlation in the control group was also observed for decreasing EBMs (NAA/Cr, PCr/ePP, NTP/ePP) even though higher  $R^2$  values were still observed for the “increasing” group of EBMs.

## **Conclusion**

In conclusion, early biomarkers, in particular thalamic Lac/NAA, acquired between 2 and 4 hours after hypoxia-ischemia predicts the MRS and histological outcomes at 48 hours. The ability to identify hypoxic-ischemic damage and predict the outcome at this early time point opens exciting perspectives for future neuroprotective trials. These EBMs can be used to select the ideal candidates for such trials. When acquired before the onset of treatment, EBMs can reduce the variability of outcome.

**Chapter 4 -The Predictive Value of late  
MRS Biomarkers (acquired at 40-48 hours  
after HI), in a piglet model of perinatal  
asphyxia**

## **Introduction**

Adverse outcomes were observed in up to 50% of cooled infants after therapeutic hypothermia (Edwards, Brocklehurst et al. 2010). Hence, research experiments focus on the development of adjunct therapies that can improve the efficacy of therapeutic hypothermia. Biomarkers, which can act as surrogate end points, are needed to speed up such experiments in order to avoid long delay of waiting for long term outcome. MRI and MRS can provide such biomarkers. MRI has assessed therapeutic hypothermia efficacy in some recent studies (Porter, Counsell et al. 2010, Rutherford, Ramenghi et al. 2010)

A recent meta-analysis of cerebral biomarkers, covering 52 studies and including 860 babies with NE managed without hypothermia therapy, concluded that early conventional MRI ineffectively identified babies at risk of long term adverse outcome and that the most accurate predictor was the deep gray matter lactate/N-acetylaspartate (Lac/NAA) peak-area ratio determined by proton ( $^1\text{H}$ ) magnetic resonance spectroscopy (MRS) (Thayyil, Chandrasekaran et al. 2010). However, currently there have been no studies, which explored the neuroprotective effects on the predictive value of these MRS biomarkers.

Our aim was to investigate the  $^1\text{H}$  and  $^{31}\text{P}$  MRS biomarkers, acquired at 40-48 hours after hypoxia-ischemia, and their correlation to histological outcome in our piglet model of HIE. Our aim was also to assess the neuroprotective effects on the correlation of MRS biomarkers with cell death.

## **Materials and methods**

Experiments were carried out according to United Kingdom Home Office guidelines. Large-white male piglets, aged < 24h were anaesthetised and surgically prepared as

described previously (Lorek, Takei et al. 1994, Faulkner, Bainbridge et al. 2011). All physiological monitoring and experimental procedures were carried out as explained previously (Faulkner, Bainbridge et al. 2011). All data used in the current study were from two large translational neuroprotection studies in a piglet model of hypoxic-ischemia as described in chapters 3 and 4. The first study explored the combination of hypothermia and Xenon (Xe/HT)(Faulkner, Bainbridge et al. 2011), the second (Robertson et al., in press) the treatment with Methyl-Isobutyl-Amiloride (MIA). MRS acquisition and analysis, statistics, histological staining and quantification and hypoxia-ischemia were as described in previous chapters. Voxel position and representative spectra for naïve and untreated animals after HI at 48 hours are shown in figure 4.1.

### **Analysis of Biomarkers and Outcome data**

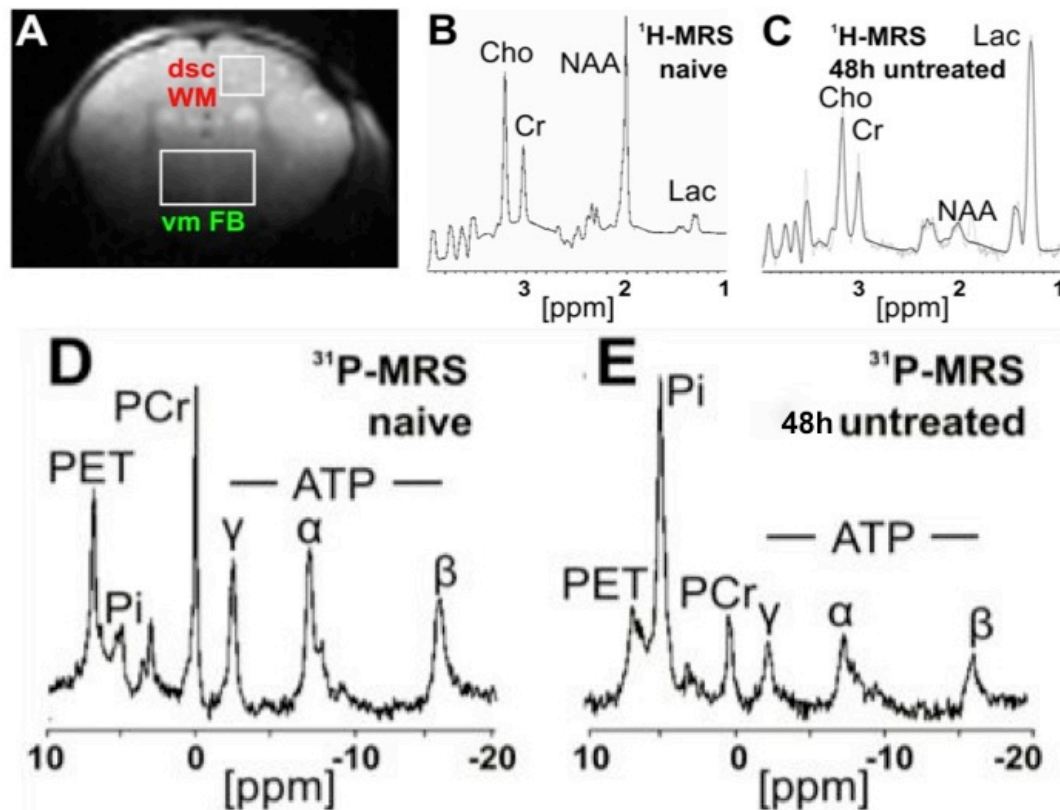
For biomarkers, we used the MRS data from the final time point, which is acquired 40 - 48h after completion of HI insults (Table 4.1). These data include  $^1\text{H}$  MRS WM and thalamic voxels (Lac/Cr, NAA/Cr and Lac/NAA), and the whole brain  $^{31}\text{P}$  MRS voxel (Pi/ePP, PCr/ePP and NTP/ePP). Cho/Cr was not included in the analysis as it demonstrated poor correlation with the outcome in our previous study (Faulkner, Bainbridge et al. 2011). All these 9 markers are described as MRS biomarkers.

We used MRS data acquired at 48 hours after HI, rather than AUC (which describes the change over the course of 48 hours), because of several reasons. Firstly, 48-hour data is a single time point data that is not altered by statistical methods. Secondly, this data is also not altered by variations that arise due to systemic changes at multiple time points. Finally, this is more relevant for clinical translation as it is not easy to acquire MRS data on multiple occasions.

Experimental study	Group	<sup>31</sup> P-Whole Brain Time in hours Mean $\pm$ SEM (n)	<sup>1</sup> H-WM voxel Time in hours Mean $\pm$ SEM (n)	<sup>1</sup> H-thalamic voxel Time in hours Mean $\pm$ SEM (n)
<b>Xe/HT study</b>	Control	47 $\pm$ 0.23 (8)	42 $\pm$ 1.1 (8)	42 $\pm$ 0.67 (8)
	Xenon	47 $\pm$ 0.41 (8)	45 $\pm$ 0.77 (8)	44 $\pm$ 0.76 (8)
	Hypothermia	47.5 $\pm$ 0.33 (7)	44 $\pm$ 0.93 (7)	43.5 $\pm$ 0.76 (7)
	Xe + Hypothermia	46 $\pm$ 1.0 (8)	43.5 $\pm$ 1.5 (8)	42.5 $\pm$ 1.4 (8)
<b>MIA study</b>	Control	47 $\pm$ 0.35 (9)	43 $\pm$ 0.51 (9)	41.7 $\pm$ 0.54 (9)
	MIA	48 $\pm$ 0.3 (8)	44 $\pm$ 0.62 (8)	43 $\pm$ 0.65 (8)

**Table 4.1:** Acquisition time in hours after HI for the final proton (<sup>1</sup>H) and phosphorus (<sup>31</sup>P) MRS data acquisition in Xe/HT and MIA experiments.

For outcome, we used histology data; density of TUNEL positive nuclei, density of cleaved caspase 3 positive cells, and the ramification index. However, caspase 3 positive cells demonstrated very poor correlation with our previous studies, and it also showed poor correlation with MRS biomarkers on initial analysis in this study. Individual histopathology outcomes for each animal and staining were combined by using an average across all regions, with the intention to reduce the number of outcomes to 2 for each animal. Previous analysis revealed that these averaged histopathology values exhibited considerably higher correlation with the biomarker AUCs than those observed for region-specific histopathology (Faulkner, Bainbridge et al. 2011).



**Figure 4.1:** **A:** Coronal NMR Image of the piglet showing the location of spectroscopy voxels in the dorsal subcortical (dsc) white matter and in ventromedial forebrain. **B and C:**  $^1\text{H}$  Magnetic resonance spectroscopy in the dorsal subcortical voxel in naïve piglet (B) and 48h after a transient, global hypoxic ischemic insult (C). Note the massive increase in the lactate peak (Lac) at 1.3ppm and the reduction of that for N-Acetyl Aspartate (NAA) at 2ppm. The 3ppm creatine (Cr) peak remains largely unchanged. **D and E:**  $^{31}\text{P}$  spectra from a naïve piglet (D) and 48 h after hypoxia-ischaemia (E). Note the increased inorganic phosphate (Pi) and decreased phosphocreatine (PCr), phosphoethanolamine (PET) and c, a and b adenosine triphosphate (ATP) in (E).

## Results

Five animals from Xenon study and one animal from MIA study died early and so are not included in final analysis. In each animal, MRS biomarkers using  $^1\text{H}$  thalamic voxel,  $^1\text{H}$  WM voxel, and whole brain  $^{31}\text{P}$  MRS were acquired serially.  $^{31}\text{P}$  MRS-derived EBMs were obtained between 46 and 48 hours after HI insult, those for the  $^1\text{H}$  thalamic voxel at 42 and 44 hours, and for the  $^1\text{H}$  WM at 42 and 45 hours in Xe/HT study. The mean and standard error of the mean for the acquisition time point for Xe/HT and MIA studies are shown in table 4.1. There was no significant difference in the acquisition time point for all biomarkers between the subgroups in both studies.

### Effect of Neuroprotection

The mean values of late biomarkers peak area ratios in both Xe/HT and MIA study groups are shown in Table 4.2 and 4.3. There is a significant difference in the values between control and treatment groups in both studies (Faulkner, Bainbridge et al. 2011, Robertson, Kato et al. 2013), representing the effect of treatment.

Histologically, the reduction of Tunel density and caspase positive cells, and increase in ramification of microglia are observed in the treated groups of both studies (figure 4.2 and 4.3)

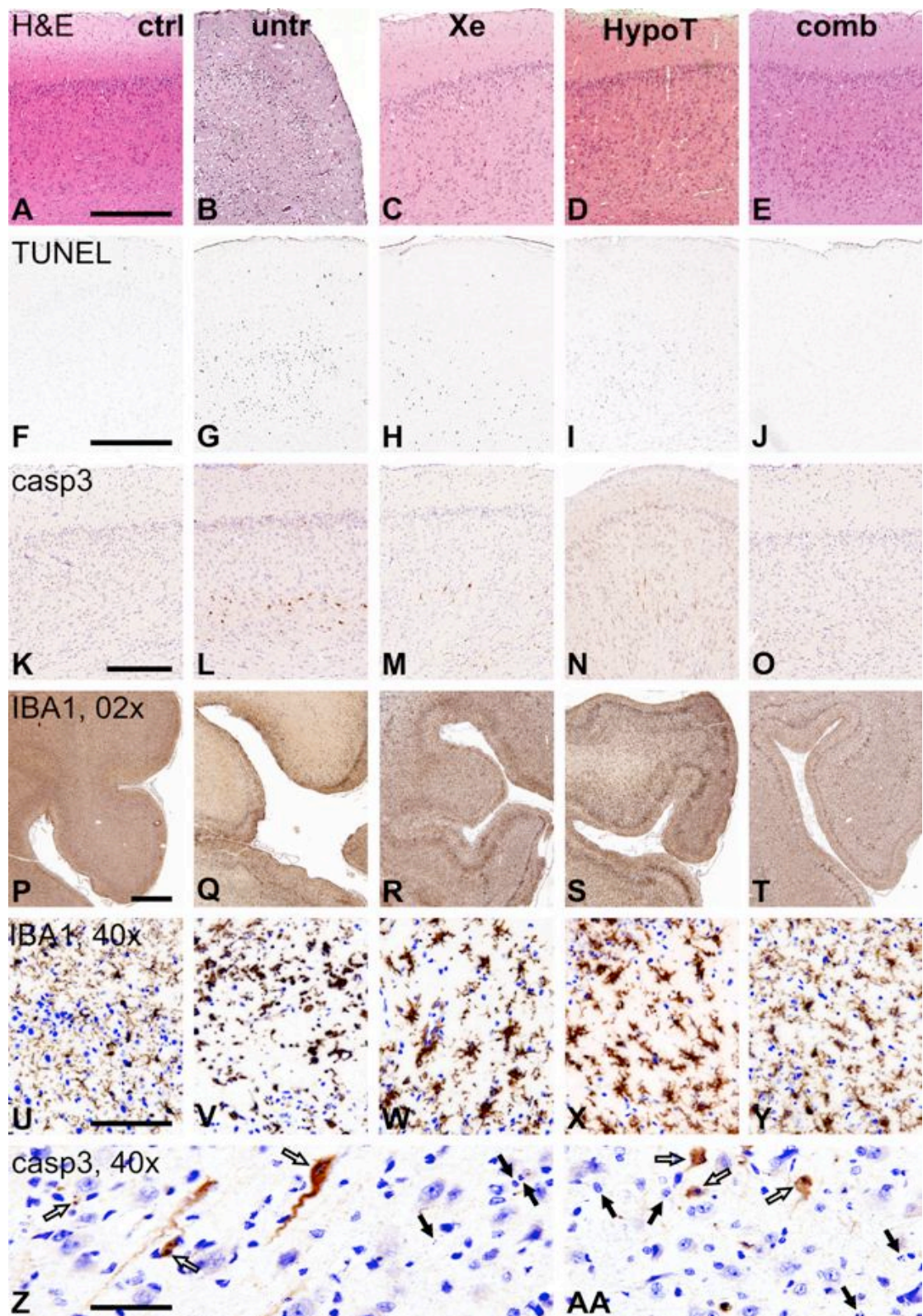
<b>Late Biomarkers</b>	<b>Control Mean (SD)</b>	<b>Xenon Mean (SD)</b>	<b>Hypothermia Mean (SD)</b>	<b>Xe/HT Mean (SD)</b>
Thalamic Lac/NAA	4.78 (12.4)	0.46 (0.48)	0.28 (0.18)	0.34 (0.52)
Thalamic Lac/Cr	1.22 (1.30)	0.67 (0.53)	0.51 (0.25)	0.49 (0.48)
Thalamic NAA/Cr	1.37 (0.58)	1.71 (0.35)	1.93 (0.35)	1.83 (0.36)
WM Lac/NAA	3.77 (7.4)	0.80 (1.15)	0.50 (0.70)	0.17 (0.07)
WM Lac/Cr	1.81 (1.56)	0.88 (0.87)	0.78 (0.84)	0.37 (0.09)
WM NAA/Cr	1.54 (0.82)	1.82 (0.75)	2.28 (0.70)	2.21 (0.49)
Pi/EPP	0.22 (0.06)	0.20 (0.07)	0.16 (0.06)	0.14 (0.03)
PCr/EPP	0.27 (0.03)	0.27 (0.04)	0.29 (0.03)	0.31 (0.02)
NTP/EPP	0.26 (0.02)	0.26 (0.02)	0.28 (0.02)	0.28 (0.01)

**Table 4.2:** Mean and standard deviation of late biomarkers peak area ratio of all the groups in Xe/HT study.



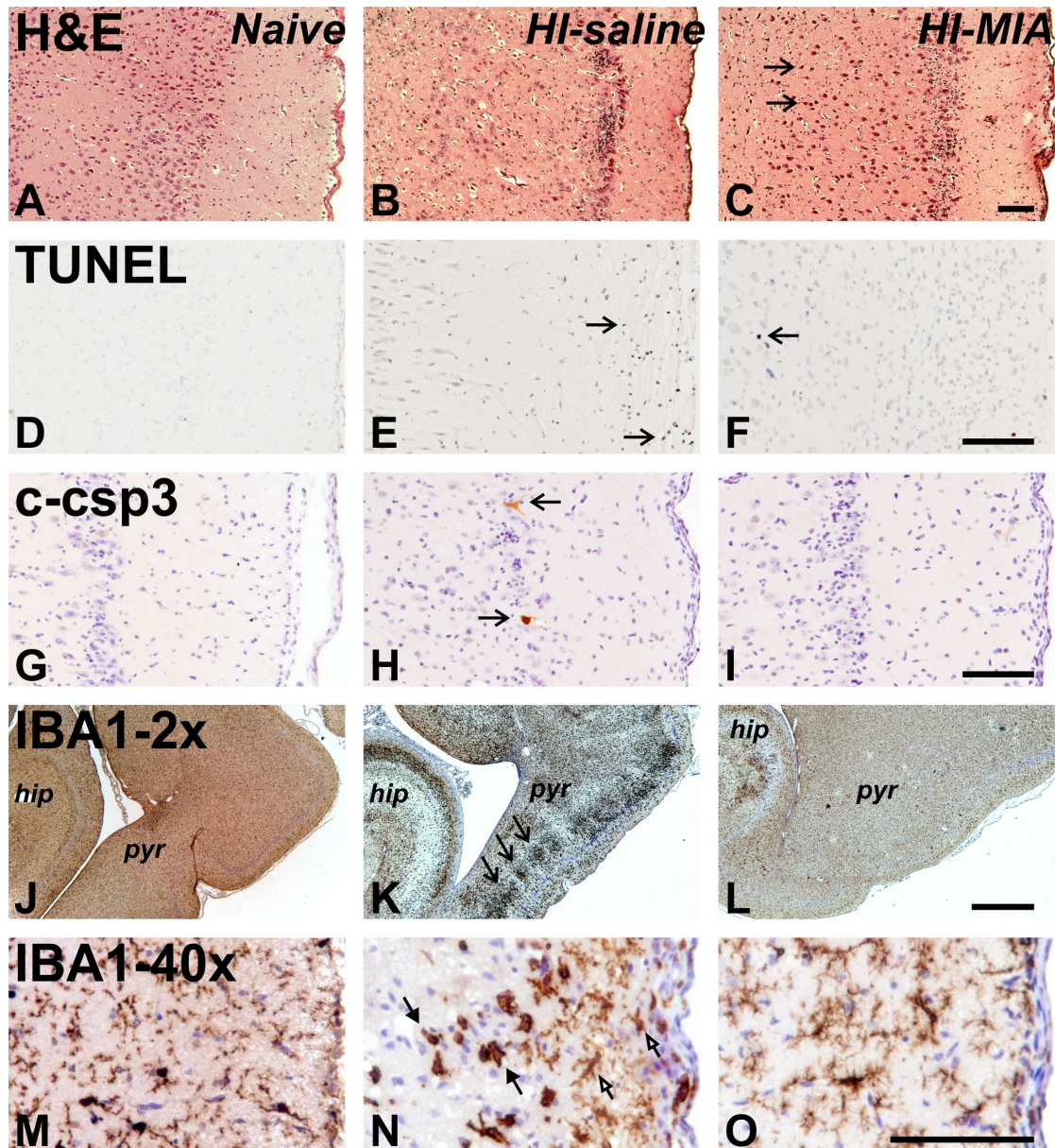
<b>Late Biomarkers</b>	<b>Control Mean (SD)</b>	<b>MIA Mean (SD)</b>
Thalamic Lac/NAA	59.99(111)	1.59 (2.48)
Thalamic Lac/Cr	2.92(2.45)	1.27 (1.33)
Thalamic NAA/Cr	0.82 (0.67)	1.30 (0.46)
WM Lac/NAA	12.16 (11)	4.9 (5.1)
WM Lac/Cr	4.77 (3.7)	3.01 (2.17)
WM NAA/Cr	0.63 (0.04)	0.99 (0.52)
Pi/EPP	0.57(0.30)	0.40(0.27)
PCr/EPP	0.13(0.09)	0.19 (0.09)
NTP/EPP	0.16 (0.11)	0.21 (0.09)

**Table 4.3:** Mean and standard deviation of late biomarkers peak area ratio of all the groups in MIA study.



**Figure 4.2:** Combination treatment decreases histological damage. Brain histology (A-E), TUNEL (F-J) and immunohistochemistry for caspase 3 (K-O, Z-AA) and IBA1 (P-Y) in naïve, control animals (A,F,K,P,U), 48 hours after untreated hypoxic ischemic insult (B,G,L,Q,V), hypothermia (C,H,M,R,W), xenon (D,I,N,S,X) and combination of xenon and hypothermia (E,J,O,T,Y). Compared with control animals in the left column, the untreated hypoxic ischemic group shows the appearance of a large number of eosinophilic, dead neurons (B), many TUNEL-positive (G) and caspase immunoreactive neurons (L), and a redistribution of microglial IBA1 immunoreactivity from a very uniform, diffuse pattern (P) to a retraction from the deep subcortical areas and clustering in superficial layer 2 and layer 4 (Q). At high magnification, the untreated group (V) shows loss of microglial branching and transformation into rounded macrophages. The changes are to a large extent reversed following combined treatment, histology in the hypothermia alone and xenon alone groups shows an intermediate phenotype.(Z,AA): High magnification of cleaved caspase 3 immunoreactivity and haematoxylin counterstain. There are numerous neurons with strong cytoplasmic immunoreactivity and pyknotic or fragmented nuclei (white arrows), but there also many cells with fragmented or pyknotic nuclei without caspase 3 immunoreactivity. Scale bars: A (A-E) - 0.5mm, F (F-J) - 0.5mm, K (K-O) - 0.5mm, P (P-T) - 1mm, U (U-Y) - 0.1mm, Z (Z,AA) - 50um (magnification for all micrographs for the same row is kept constant). H&E: hematoxylin and eosin. Permission to reproduce this figure has been granted by John Wiley and Sons





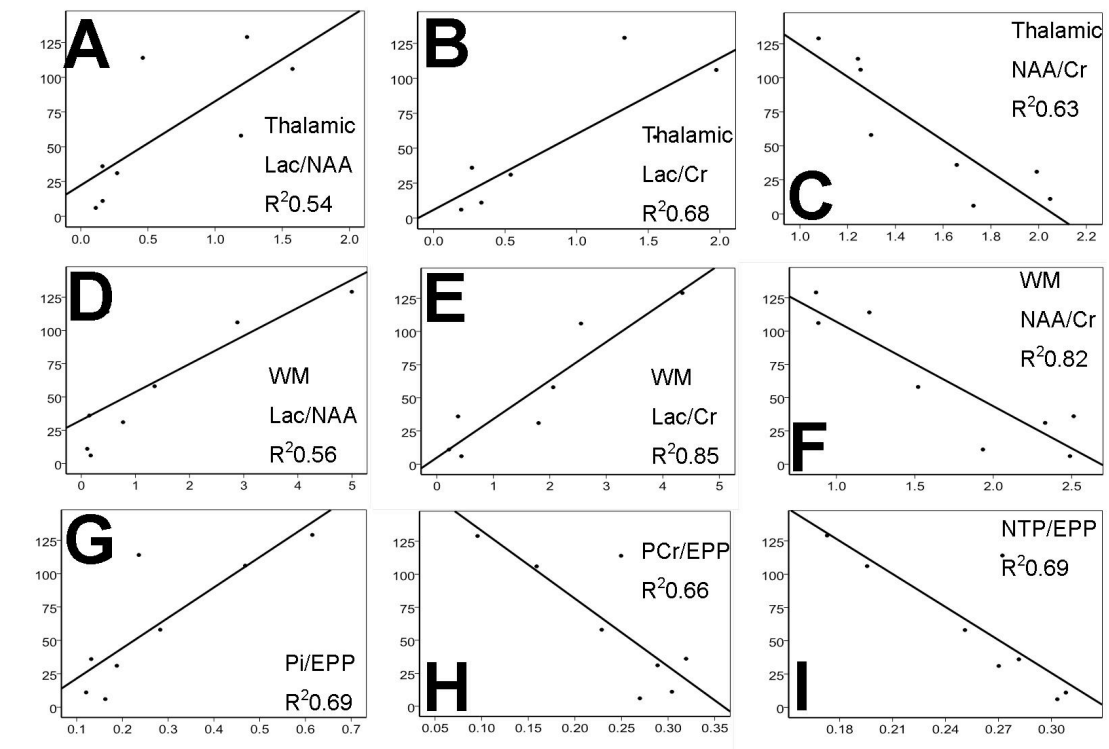
**Figure 4.3:** Methyl isobutyl amiloride (MIA) treatment decreases histological damage. Haematoxylin-eosin (H&E) histology (a–c), transferase mediated biotinylated d-UTP nick end-labelling (TUNEL) (d–f) and immunohistochemistry for caspase 3 (g–i) and ionized calcium- binding adaptor molecule1 (IBA1)(j–o) in naive animals (a,d,g,j,m), and 48 h after hypoxia-ischaemia for placebo (b, e, h, k, n), and for post-insult MIA (c, f, i, l, o). Compared with naive (left column), placebo animals lost deeper layer neurons on H&E (b), and had a TUNEL+ cell preponderance (e), occasional caspase 3 immunoreactive neurons (h, arrows), and a redistribution of microglial IBA1 immunoreactivity from a very uniform, diffuse pattern (j) to a retraction from deep subcortical areas and large columnar clusters around layer 4 (k, arrows). At high magnification, the placebo group (n) also shows loss of microglial branching and microglial transformation into rounded macrophages (arrows). The cortical changes are to a large extent reversed by MIA, but the hippocampal dentate gyrus (hip) appears unaffected. Permission to reproduce this figure has been granted by John Wiley and Sons.

### **Correlation of MRS biomarkers and Histopathology Outcomes for untreated animals in the Xe/HT study**

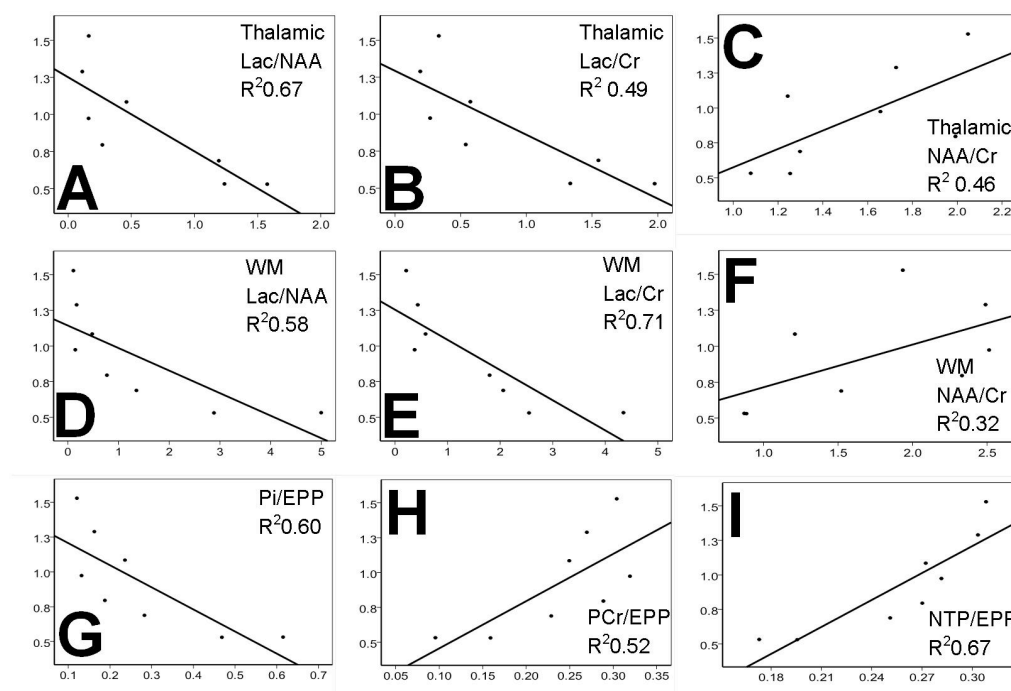
The results of regression analysis between MRS biomarkers and histopathology outcome are shown in table 4.4. On regression analysis, all MRS biomarkers showed significant correlation with Tunel positive cell death and microglial ramification index. WM Lac/Cr showed highest and significant correlation with Tunel cell death ( $R^2=0.85$ ,  $p = 0.003$ ), whereas NTP/EPP ( $R^2=0.78$ ,  $p = 0.004$ ) demonstrated highest and significant correlation with ramification index. Thalamic and WM Lac/NAA, WM NAA/Cr, Pi/EPP and PCr/EPP also demonstrated good significant correlation with both outcome measures. However, WM NAA/Cr showed poor correlation with ramification index ( $R^2=0.32$ ,  $p = 0.14$ ). All correlation graphs were shown in figures 4.4 and 4.5.

<b>Biomarker</b>	<b>Tunel L - Correlation (R<sup>2</sup>)</b>	<b>p - value</b>	<b>Iba1 L - Correlation (R<sup>2</sup>)</b>	<b>p - value</b>
thLac/NAA	0.54	<b>0.03</b>	0.67	<b>0.01</b>
thLac/Cr	0.68	<b>0.02</b>	0.49	<b>0.03</b>
thNAA/Cr	0.63	<b>0.01</b>	0.46	<b>0.04</b>
wmLac/NAA	0.56	<b>0.03</b>	0.58	<b>0.02</b>
wmLac/Cr	0.85	<b>0.003</b>	0.71	<b>0.008</b>
wmNAA/Cr	0.82	<b>0.002</b>	<b>0.32</b>	<b>0.140</b>
Pi/EPP	0.69	<b>0.01</b>	0.60	<b>0.02</b>
Pcr/EPP	0.66	<b>0.01</b>	0.52	<b>0.04</b>
NTP/EPP	0.69	<b>0.01</b>	0.78	<b>0.004</b>

**Table 4.4:** Correlation of late MRS biomarker, acquired at around 48 hours after HI, with histological outcome from the control group in Xe/HT study.



**Figure 4.4:** Correlation between late MRS biomarkers (x axis) and TUNEL positive cell death (y axis) and their  $R^2$  values from control group in Xe/HT study

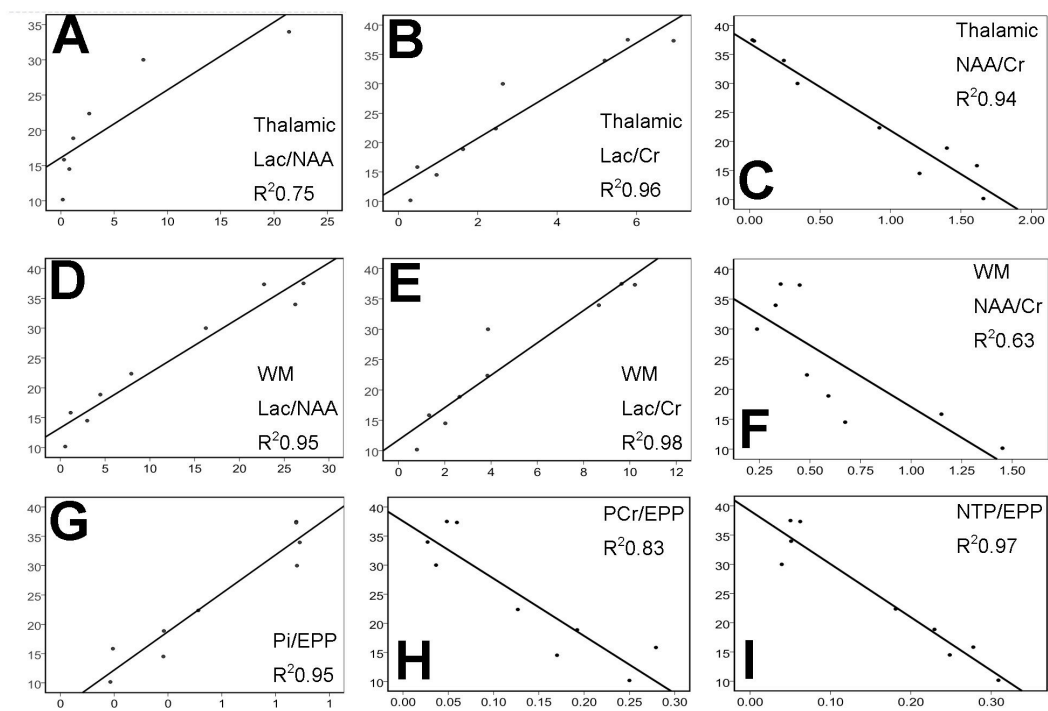


**Figure 4.5:** Correlation between late MRS biomarkers (x axis) and microglial ramification index (y axis) and their  $R^2$  values from control group in Xe/HT study

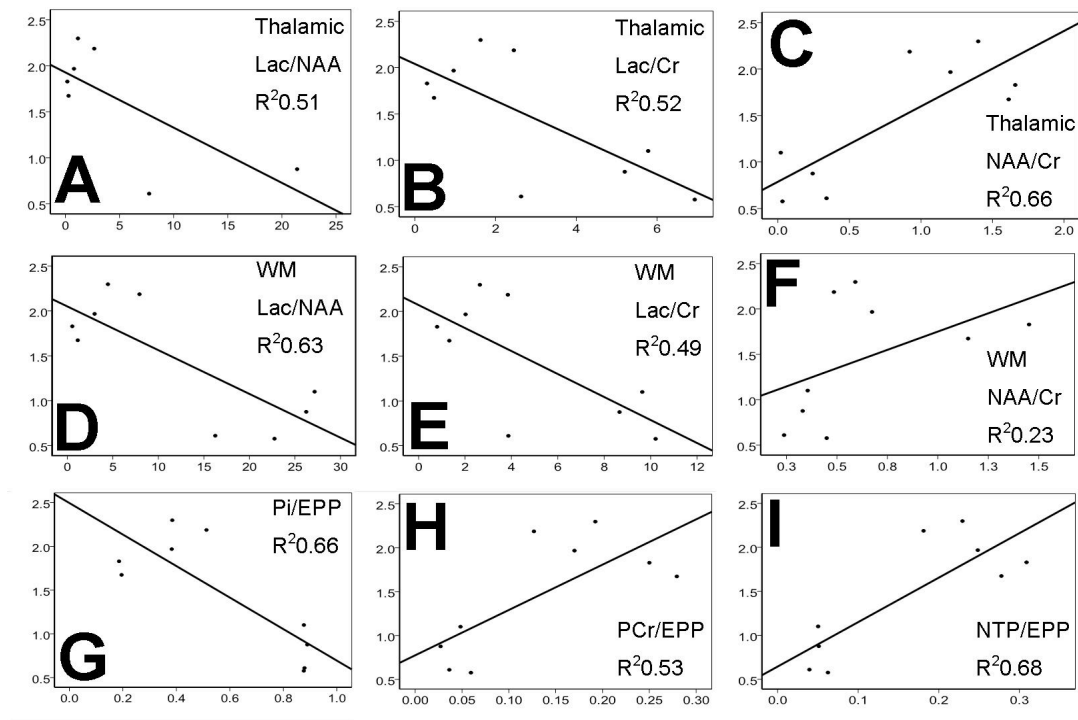


### **Correlation of MRS biomarkers and Histopathology Outcomes for untreated animals in the MIA study**

To revalidate the predictive value of MRS biomarkers, the same analysis was done using the data from the MIA treatment study in a more severe form of HI insult. All MRS biomarkers showed significant correlation with Tunel positive cell death and ramification index, as shown in figure 4.6 and 4.7. The results of the regression analysis are shown in table 4.5. Similar to Xe/HT study, WM Lac/Cr demonstrated highest and positive correlation with Tunel positive cell death ( $R^2 = 0.98$ ,  $p = 0.00$ ) and NTP/EPP with microglial ramification ( $R^2 = 0.68$ ,  $p = 0.006$ ). Compared to Xe/HT study results, all 9 markers showed better correlation for Tunel positive cell death with statistical significance ( $p < 0.01$ ); the  $R^2$  of 6 out of 9 biomarkers were more than 0.90 (Table 4.5). Similarly better significant correlation was noted with ramification index. However WM NAA/Cr demonstrated less and non-significant correlation with ramification index, similar observation as in Xe/HT study.



**Figure 4.6:** Correlation between late MRS biomarkers (x axis) and TUNEL positive cell death (y axis) and their  $R^2$  values from control group in MIA study



**Figure 4.7:** Correlation between late MRS biomarkers (x axis) and microglial ramification index (y axis) and their  $R^2$  values from control group in MIA study

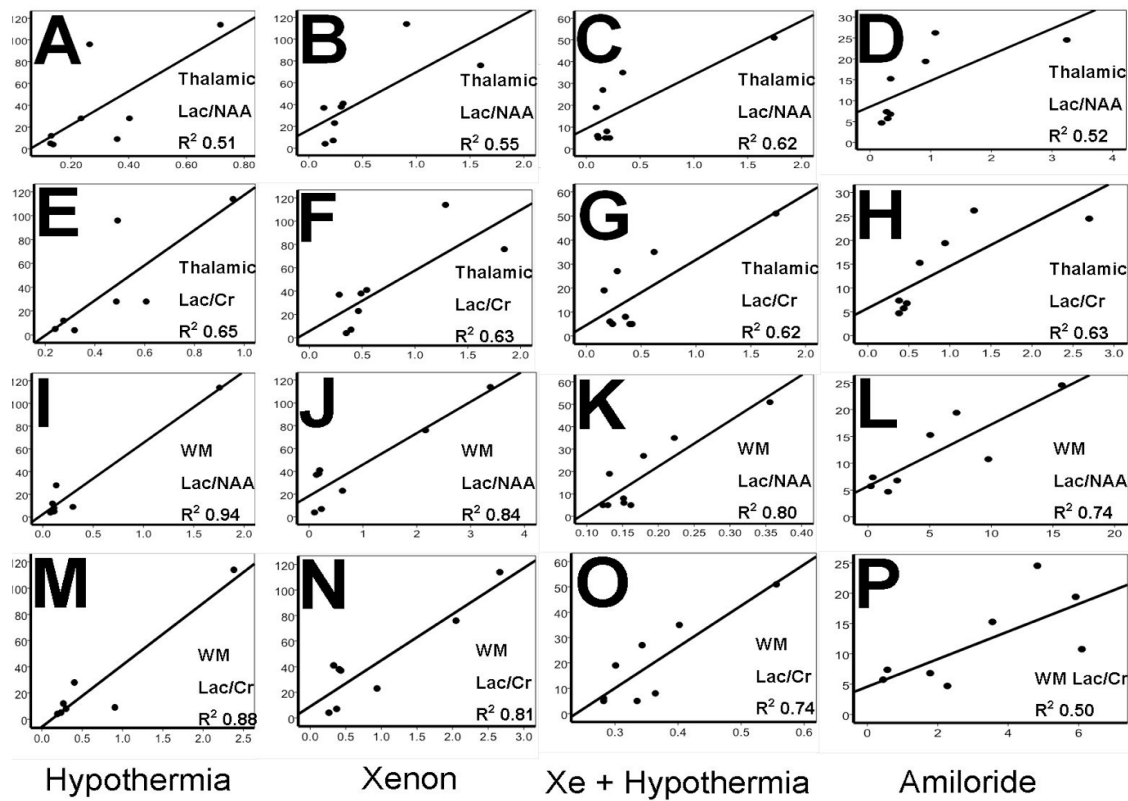
<b>Biomarker</b>	<b>Tunel L - Correlation (R<sup>2</sup>)</b>	<b>p - value</b>	<b>Iba1 L - Correlation (R<sup>2</sup>)</b>	<b>p - value</b>
thLac/NAA	0.75	<b>0.01</b>	0.51	<b>0.07</b>
thLac/Cr	0.96	<b>&lt;0.001</b>	0.52	<b>0.02</b>
thNAA/Cr	0.94	<b>&lt;0.001</b>	0.66	<b>0.008</b>
wmLac/NAA	0.95	<b>&lt;0.001</b>	0.63	<b>0.01</b>
wmLac/Cr	0.98	<b>&lt;0.001</b>	0.49	<b>0.03</b>
wmNAA/Cr	0.63	<b>0.01</b>	<b>0.23</b>	<b>0.18</b>
Pi/EPP	0.95	<b>&lt;0.001</b>	0.66	<b>0.007</b>
PCr/EPP	0.83	<b>0.002</b>	0.53	<b>0.02</b>
NTP/EPP	0.97	<b>&lt;0.001</b>	0.68	<b>0.006</b>

**Table 4.5:** Correlation of late MRS biomarkers with histological outcome from control group in MIA study

### **Correlation of MRS biomarkers after neuroprotective intervention in Xe/HT and MIA studies**

As there was a consistent, significant and positive correlation for most MRS biomarkers with histopathology outcomes in the control group, we further analysed them in the intervention groups (Xenon, hypothermia and combined Xenon and hypothermia) of Xe/HT study and treatment group in MIA study. Table 4.6 shows all the correlation values of MRS biomarkers with Tunel positive cell death. All MRS biomarkers showed significant correlation with Tunel positive cell death (Table 4.6). WM Lac/NAA showed highest and significant correlation across all treatment groups,

particularly very high correlation for hypothermia group ( $R^2 = 0.94$ ,  $p = 0.001$ ). Thalamic Lac/NAA and Lac/Cr, WM Lac/Cr also showed significant correlation with TUNEL positive cell death, consistently observed in all intervention groups (Figure 4.8). Pi/EPP and WM NAA/Cr showed good correlation too. NTP/EPP and PCr/EPP demonstrated good correlation in hypothermia and Xenon groups; however, there was poor correlation in the combined xenon and hypothermia group. Thalamic NAA/Cr showed good correlation in xenon and combined group; however the correlation was non-significant in hypothermia and MIA treated groups. All MRS biomarkers showed poor and non-significant correlation with microglial ramification index across all treatment groups.



**Figure 4.8:** Correlation between late MRS biomarkers (x axis) and TUNEL positive cell death (y axis) and their  $R^2$  values from treatment groups in Xe/HT and MIA studies.

<b>Late Biomarkers</b>	<b>Hypothermia R<sup>2</sup> (p value)</b>	<b>Xenon R<sup>2</sup> (p value)</b>	<b>Xenon + Hypothermia R<sup>2</sup> (p value)</b>	<b>Amiloride R<sup>2</sup> (p value)</b>
<b>thLac/NAA</b>	0.51 (0.02)	0.55 (0.02)	0.62 (0.01)	0.52 (0.01)
<b>thLac/Cr</b>	0.65 (0.02)	0.63 (0.01)	0.62 (0.01)	0.63 (0.02)
<b>thNAA/Cr</b>	0.43 (0.05)	<b>0.40 (0.06)</b>	0.70 (0.005)	0.45 (0.01)
<b>wmLac/NAA</b>	0.94 (0.001)	0.84 (0.01)	0.80 (0.001)	0.74 (0.002)
<b>wmLac/Cr</b>	0.88 (0.001)	0.81 (0.001)	0.74 (0.01)	0.50 (0.04)
<b>wmNAA/Cr</b>	0.83 (0.01)	0.61 (0.01)	0.52 (0.02)	0.52 (0.03)
<b>Pi/EPP</b>	0.59 (0.01)	0.90 (<0.001)	0.59 (0.02)	0.56 (0.02)
<b>Pcr/EPP</b>	<b>0.27 (0.19)</b>	0.70 (0.009)	<b>0.26 (0.16)</b>	0.64 (0.03)
<b>NTP/EPP</b>	0.52 (0.04)	0.79 (0.003)	<b>0.03 (0.6)</b>	0.51 (0.01)

**Table 4.6:** Correlation between late MRS biomarkers and Tunel positive cell death from treatment groups in Xe/HT and MIA studies.

## Discussion

This study has shown that all MRS biomarkers, acquired at around 48 hours after hypoxic-ischemic insult, demonstrated significant correlation with the histopathological outcomes after HI. It has also shown that this correlation remains even after neuroprotective intervention. Out of all the MRS biomarkers, thalamic and WM Lac/NAA, thalamic and WM Lac/Cr and Pi/EPP showed a significant correlation with histological outcome in both untreated and treated animals in both studies. There are not many studies in the literature that correlated Lac/NAA to Tunel density representing cell death. The existence of significant correlation of Lac/NNA to

histological outcome after therapeutic intervention in animals after HI has not been shown before.

This consistently high and significant correlation of thalamic and WM Lac/NAA with histological outcome observed in control animals of our study may be due to delayed Lac increase and fall in NAA which happens around 48 hours after HI when the MRS biomarkers acquired. This significant correlation was also present in treated animals those received any form of neuroprotective intervention. Our studies showed that neuroprotective treatment ameliorates the cerebral Lac rise and fall in NAA, as well as reducing tissue damage (Faulkner, Bainbridge et al. 2011, Robertson, Kato et al. 2013). Thus thalamic and WM Lac/NAA correlated well with histological cell death. This significant correlation combined with treatment effect clearly explains the biological basis of late biomarkers, particularly thalamic and WM Lac/NAA.

Of the  $^{31}\text{P}$ -MRS, our study revealed that Pi/EPP had the highest correlation with outcome, followed by PCr/EPP and NTP/EPP. Pi/EPP had a significant correlation with outcome after all intervention in all forms of injury, but PCr/EPP and NTP/EPP did not showed significant correlation in combined xenon and hypothermia group.

The results of this study did not change with the severity of injury. In the previous chapter, early biomarkers demonstrated differential correlation depending on the severity of HI. Increasing EBMs like Lac/NAA, Lac/Cr showed significant correlation in moderate and severe injury as in Xe/HT study and MIA study respectively; however decreasing EBMs like NAA/Cr, NTP/EPP showed significant correlation only in severe injury as in MIA study. This does not hold true with this study, as MRS biomarkers, all increasing and decreasing types, acquired at 48 hours showed significant correlation with the outcome in moderate and severe injury.



Microglia are highly ramified cells in the resting state (Raivich 2005). When activated by ischemia or infection, microglia can take on a deramified, amoeboid phagocytic phenotype. Microglia are activated along with neutrophil infiltration, during cerebral hypoxia-ischemia and reperfusion (Hudome, Palmer et al. 1997). Activated microglia were observed from 4 hours after reperfusion and continued to increase over the next 48 hours (Bona, Andersson et al. 1999). An association has been shown between the activation, migration and proliferative activity of brain microglia 1-3 days after the injury with brain lactate (Garcia JH and Y 1974). Microglial response to injury was assessed by the microglial ramification index in this study. From this study, increasing biomarkers including Lac/NAA correlated negatively with ramification index in control animal and decreasing biomarkers correlated positively with it; however none of the biomarkers correlated with ramification index in treated animals. This could be due to the fact that microglia were still undergoing the changes of activation and proliferation at 48 hours after HI. Although ramification was reversed in combined treatment with xenon and hypothermia, there were intermediate changes in other intervention groups (Faulkner, Bainbridge et al. 2011).

Therapeutic hypothermia for newborn infants with neonatal encephalopathy has taken approximately 15 years to reach the clinical stage in the developed world (Edwards 2009). As several promising neuroprotective agents are being tested currently (Kelen and Robertson 2010), these therapies need more rapid clinical translation, for which robust and sensitive quantitative biomarkers predicting long-term outcome are required. More importantly, these biomarkers should predict outcome after neuroprotective intervention.

## **Conclusion**

In this study, most MRS peak area ratios, particularly Lac/NAA and Lac/Cr, have shown good and significant correlation with the outcome after neuroprotective intervention independent of severity of HI. Their prognostic utility should be evaluated in newborn infants with HI after neuroprotective intervention (hypothermia). These findings provide an excellent opportunity to try these biomarkers as surrogate end points on forthcoming clinical trials.

**Chapter 5 - The predictive value of MRS  
biomarkers after therapeutic hypothermia  
in neonatal infants with NE - Clinical  
Translation**

## **Introduction**

Current research focuses on the development, evaluation and deployment of adjunct therapies that can enhance the efficacy of therapeutic hypothermia. The associated clinical trials are particularly challenging and time consuming, because of necessarily large sample sizes and long follow-up delay. Robust biomarkers assessing treatment effects and predictive of long term outcome have value as surrogate endpoints for phase II randomised control trials of the most promising neuroprotectants and minimise the risk of failure in phase III randomised control trials.

In the previous 2 chapters, it has been shown that early and late MRS biomarkers had good significant correlation with short-term outcome following HIE in a piglet model with or without neuroprotection. In the present study we used MRI and deep gray matter MRS in babies with neonatal encephalopathy (NE) managed with and without therapeutic hypothermia to determine whether their predictive accuracies were altered by such treatment

## **Patients and methods**

We recruited all term babies with NE admitted to the tertiary neonatal unit at University College Hospital between October 2006 and September 2009 who had MRI and MRS aged 4 to 14 days.

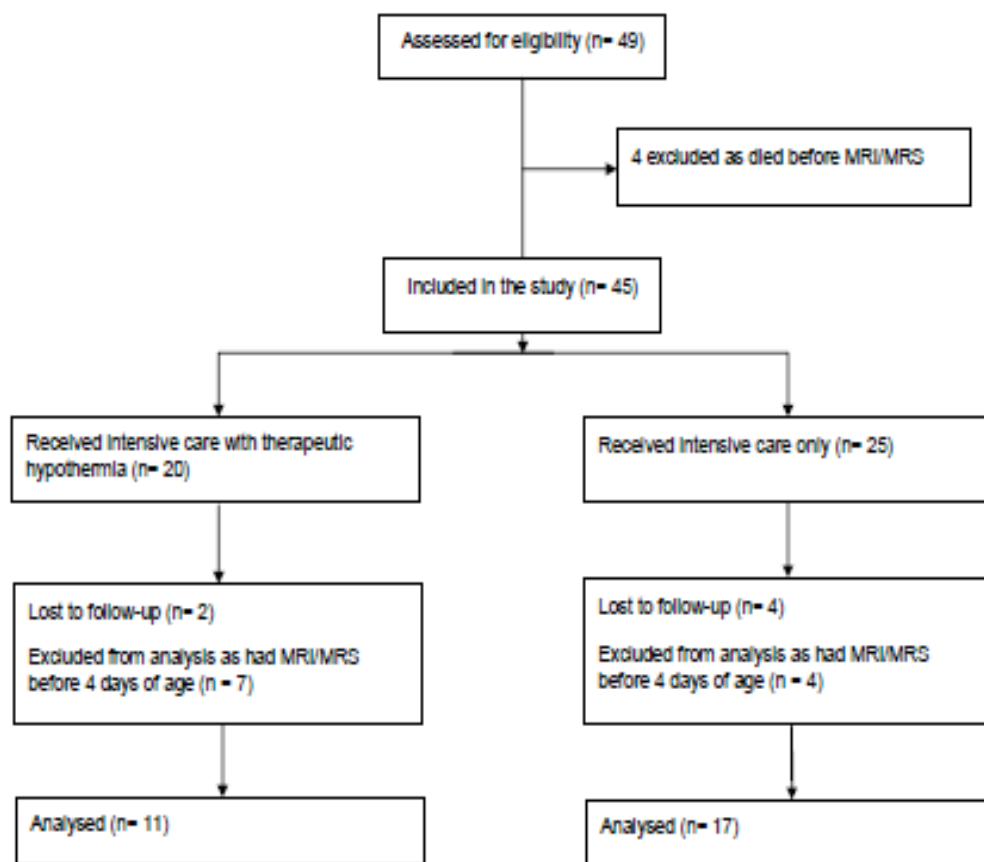
NE was diagnosed by the following criteria; (1) evidence of hypoxia during delivery consisting of at least one of: (a) Apgar score <5 10 minutes after birth, (b) continued need for resuscitation, including endotracheal or mask ventilation, 10 minutes after birth, (c) acidosis within 60 minutes of birth (defined as any umbilical cord, arterial or capillary pH <7.00) with base deficit >16mmol/L in any blood sample (arterial, venous or capillary); and (2) evidence for encephalopathy, consisting of abnormal consciousness state (lethargy, stupor or coma) and at least one of : hypotonia,

abnormal reflexes including oculomotor or pupillary abnormalities, an absent or weak suck, or clinical seizures. Figure 5.1 shows the study flow chart.

Mode of delivery, gestational age, sex, birth weight, Apgar score, birth occipitofrontal head circumference, hypothermia intervention status, Sarnat NE grading (Sarnat and Sarnat 1976) and age at MRI were recorded. The East and Central London Research Ethics Committee approved this study.

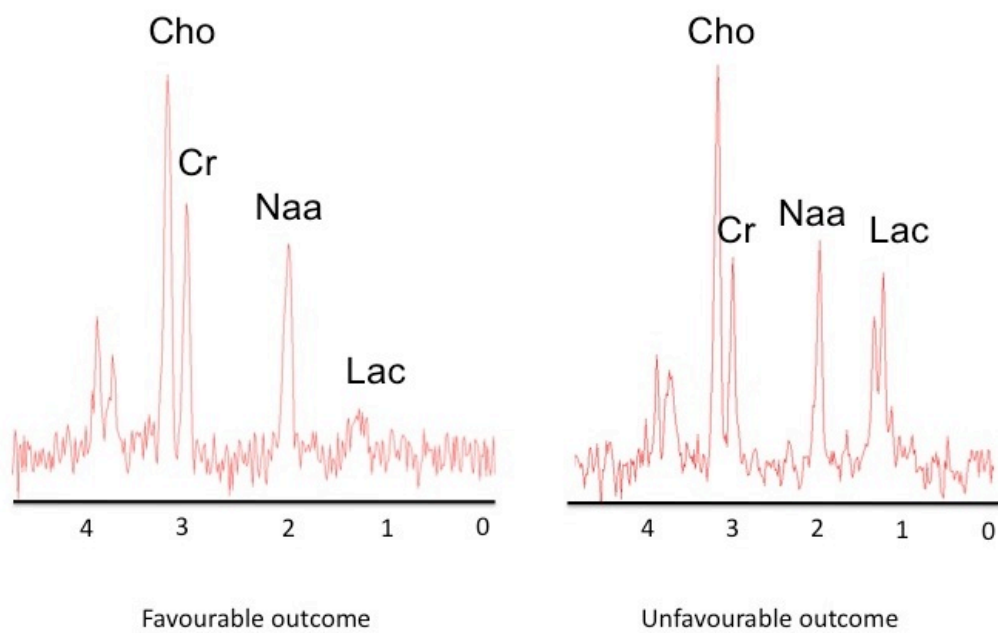
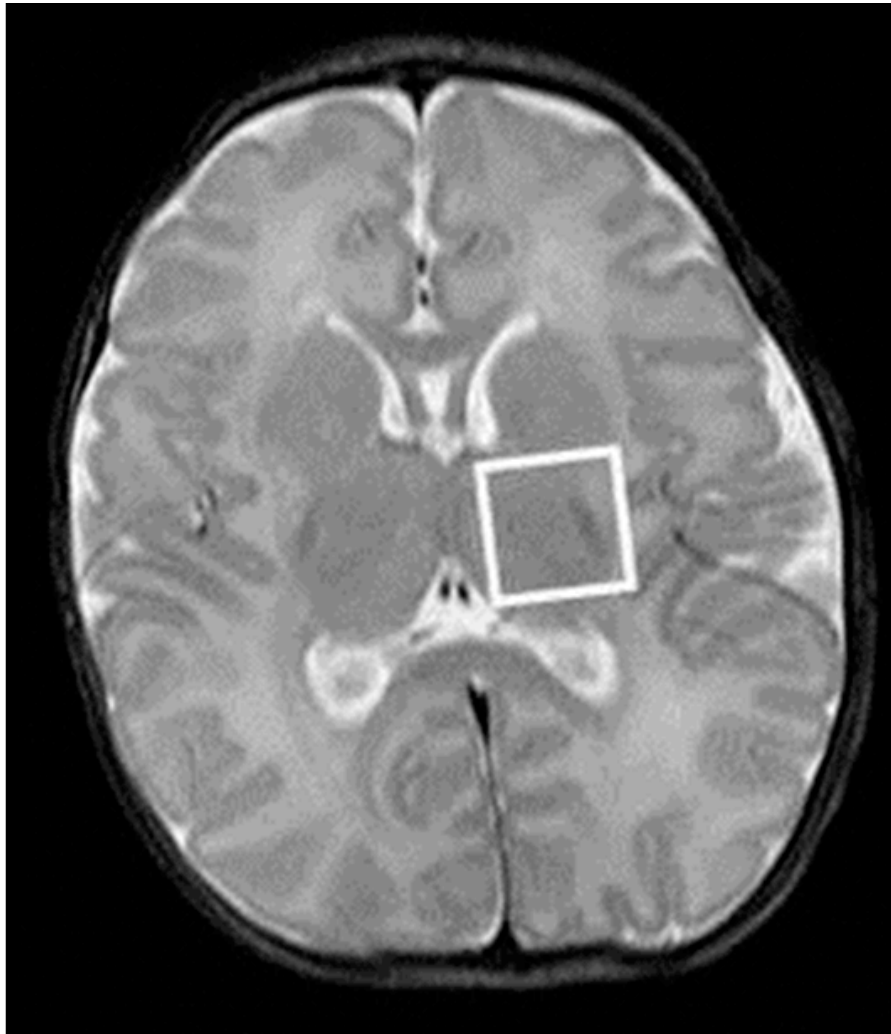
### **MRI and MRS**

MRI and MRS were performed on a 1.5-Tesla Avanto (Siemens, Erlangen, Germany). Imaging used 3D T<sub>1</sub>-weighted spoiled gradient echo (field of view (FOV) 210x158 cm, repetition time (TR) 17ms, echo time (TE) 6ms, 1 average, 0.8x0.8x1mm voxel, acquisition matrix 512x384), 2D T<sub>2</sub>-weighted fast spin echo (axial and coronal sections; FOV 210x158cm, slice thickness 3mm, acquisition matrix 512x384, 3 averages, TR 6000 ms, TE 110ms) and diffusion tensor imaging spin echo EPI sequence (30 diffusion directions, FOV 230x172.5mm, slice thickness 3 mm, acquisition matrix 128x96, 1 average, TR 5200 ms, TE 131 ms, b 600 s/mm<sup>2</sup>). Spectroscopy used 1H PRESS (TR 2290 ms, TE 288 ms, 37 summable subspectra of 8 averages each, 1.5 x 1.5 x 1.5 cm voxel centred on the left thalamus and lentiform nucleus (Figure 5. 2). Metabolite ratios including Lac/NAA, Lac/total creatine (Cr), Lac/choline (Cho), NAA/Cr and NAA/Cho were calculated using LC model as previously described (Cheong, Cady et al. 2006).



**Figure 5.1:** Study flow chart

All babies had MRI aged 4 to 14 days, and all cooled babies had rewarmed to normal temperature beforehand. MRI and MRS were performed sequentially during natural sleep or after sedation with oral chloral hydrate (50 mg/kg) for babies breathing spontaneously or with intravenous morphine infusion (10 micrograms/kg/hour) if mechanically ventilated. Babies were studied in a protective Perspex pod. For immobilization and acoustic noise reduction, the infant's head was partly surrounded by soft padding and both ears were protected with 24-dB attenuation ear plugs (Earsoft; Earo, Indianapolis, Ind) and 7-dB attenuation earmuffs (Natus minimuffs, Natus Medical Inc., San Carlos, CA). Neonatal intensive care, including mechanical ventilation, intravenous fluids, and ionotropic drug administration, was continued as appropriate, with heart rate, oxygen saturation, temperature and ECG monitoring. A neonatologist and neonatal nurse were present throughout.



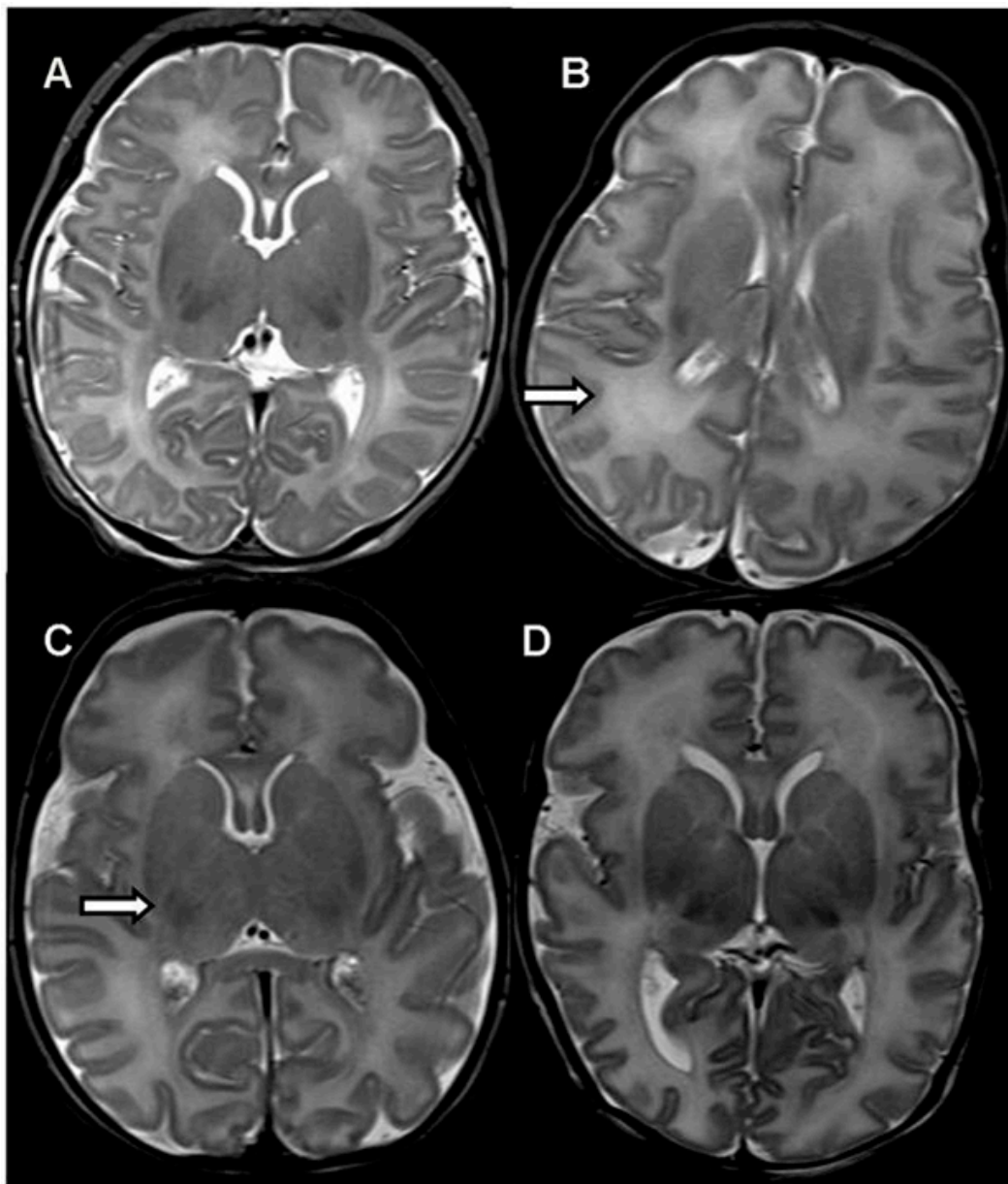
**Figure 5.2:** Thalamic voxel position (transverse plane). Typical MR spectra with normal lactate at 1.33ppm for infants with favourable outcome and raised lactate for infants with unfavourable outcome.



All MRI scans were assessed by a single pediatric neuroradiologist (4 years experience) for quality, normal anatomy and acquired lesions. As previously described (Miller, Ramaswamy et al. 2005, Rutherford, Ramenghi et al. 2010) MRI was classified as abnormal when there was at least one of: moderate or severe lesions in the basal ganglia and thalamus, or severe white matter lesions including in the watershed area (Figure 5.3). Posterior limb of internal capsule (PLIC) was classified abnormal as detailed previously (Rutherford, Ramenghi et al. 2010).

### **My Role**

I worked as a neuroimaging fellow in the neonatal unit at University College Hospital during the study period. I obtained informed consent for MRI from parents. Along with a team of advanced neonatal nurse practitioners, I stabilised all the babies and shifted them safely to the magnet room in a transport incubator, cared for them during the scan and transported them back to the unit safely. I also presented the case history of infants in the weekly neuro-meeting with a neuro-radiologist where each MRI/MRS scan results were reported. I also ensured that all infants were booked for neurodevelopmental follow up appointment. I also worked in clinical shifts as a registrar (1:9 on-call shift system) where I cared for all neonatal infants. A team of physicists helped me to collect MRS data in the study. I analysed all the data using appropriate statistical methods, later checked by the statistician.



**Figure 5.3:** T2-weighted MRI abnormalities in NE (transverse plane). . (A) Normal deep gray matter including basal ganglia and normal surrounding white matter and cortical folds. (B) Increased white-matter signal intensity (arrow) demonstrating moderate white matter lesions. (C) Abnormal low signal intensity in basal ganglia and thalamus (arrow) demonstrating local deep gray matter injury (D) Severe white matter and deep gray matter lesions in severe global hypoxic-ischemic injury.

## **Neurodevelopmental outcome**

All survivors were assessed at age 18 months by a developmental paediatrician (5 years experience) and an occupational therapist using standard neurologic assessment (Amiel-Tison.C 2001) and the Bayley Scales of Infant and Toddler Development (Bayley-3). Bayley-3 comprises 5 assessment scales covering cognitive, language (including receptive communication and expressive communication) and motor (including fine and gross motor) which are combined as a single composite score (Bayley 2006). Outcomes were categorised as favourable (normal neurologic assessment and composite score  $\geq 80$ ) or unfavourable (functional motor deficits and composite score  $< 80$  or died due to NE).

## **Statistical analysis**

We used Predictive Analytics Software 18 (PASW; IBM, USA) for all statistical analyses. Intergroup clinical and perinatal characteristics were compared by  $\chi^2$  for categorical data and Mann-Whitney test for continuous data. Predefined threshold values based on published literature were used to calculate diagnostic indices (Thayyil, Chandrasekaran et al. 2010). The efficacies of MRI and MRS for predicting abnormal outcome at age 18 months were compared by calculating their predictive accuracies, sensitivities, specificities, and positive and negative predictive values. Exact confidence intervals (CI) based on the F distribution were calculated for sensitivity, specificity and predictive values.

For direct comparison of the diagnostic utilities of individual tests, we used diagnostic odds ratios (DOR) and area under the curve (AUC). AUC was calculated using PASW 18. DOR is the ratio of the odds of positivity with disease relative to that without disease and was calculated as described in the literature (Glas, Lijmer et al. 2003). Univariate analysis assessed relationships between Apgar score, Sarnat NE

severity, hypothermia therapy, MRI and MRS results, and motor outcome. Logistic regression and calculation of adjusted odds ratios (adjusted OR, 95% CI) examined associations between MRI and MRS results and motor outcome including allowance for perinatal factors significant on univariate analysis. Two-sided  $p < 0.05$  was assumed significant.

## **Results**

Forty-nine babies were recruited of which 28 were included for final analysis (Fig. 5. 1 for exclusions and Table 5.1 for included infants' clinical characteristics). Eleven babies received therapeutic hypothermia (33.5-34°C for 72 h starting within 6 h of delivery). Birth weights, birth gestational age, Apgar scores aged 10 min and birth head circumferences were similar in both groups. Median age at MRI/MRS was 7 d in uncooled babies and 10 d in cooled. Both groups had equal numbers of infants with moderate or severe encephalopathy as assessed by Sarnat grading (Sarnat and Sarnat 1976). However, no cooled infants had mild NE whereas 6 uncooled did ( $p=0.01$ ).

## **Comparative biomarker prognostic accuracies**

Lac/NAA sensitivities and specificities for predicting adverse outcome were respectively 100% (95% CI; 55,100) and 100% (95% CI; 90, 100) in uncooled babies, and 100% (95% CI; 57, 100) and 100% (95%; 84, 100) in previously cooled (Table 5.2). However, MRI sensitivities and specificities were respectively 100% (95% CI; 48,100) and 71% (95% CI; 60,71) in uncooled babies and 66%(95% CI; 24, 93) and 75%(95% CI; 59,85) in previously cooled (Table 5. 2). Thalamic Lac/NAA and Lac/Cr were the best predictors of unfavourable outcome (Table 5.2).

The predictive accuracy (AUC) of thalamic Lac/NAA was 0.97 for uncooled and 1.0 for previously cooled babies (Table 5. 2): thalamic Lac/Cr predictive accuracies were also high in both groups. However, MRI predictive accuracy was worse. The thalamic

Lac/NAA DOR was much better than for all other predictors (Table 5.2). Importantly, there was no intergroup Lac/NAA difference between favourable and unfavourable outcome (Fig 5. 4).

On univariate analysis, Sarnat NE severity was associated with motor outcome ( $p = 0.001$ ). Hence Sarnat grading and hypothermia treatment were included as covariates in binomial logistic regression analyses. With and without adjustment for Sarnat NE grade and hypothermia treatment logistic regression showed that both Lac/NAA and Lac/Cr had similar good predictive accuracy (Table 5.3). Before adjustment for Sarnat NE grade and hypothermia, treatment MRI predicted adverse outcome: however, after adjustment, MRI OR was not statistically significant (Table 5.3).

Six babies had unfavourable outcome; three of them were cooled (Table 5.4). In the uncooled group, two babies had global developmental delay with microcephaly and one had severe hemiplegic cerebral palsy (CP). In the cooled group, one baby had deafness and gross motor delay, one had hemiplegic CP, and one had spastic CP with visual impairment. All uncooled infants with mild NE had normal outcome. All babies with unfavourable outcome had raised cerebral Lac/NAA. Although MRI correctly identified all uncooled babies with unfavourable outcome, MRI did not identify the cooled baby who developed gross motor delay.

Characteristics	Cooled (n=11) ◀	Uncooled (n=17)	P value
Median (IQR) gestational age at birth (wk)	40(39.5, 41)	40 (40, 41)	1.0
Median (IQR) birth weight (gm)	3625(3182, 4365)	3140(2660, 3580)	0.07
Median (IQR) Apgar at 10 min	5 (3.5, 7.5)	7.4 (7, 9)	0.07
Median (IQR) head circumference (cm)	36 (35.2, 36.3)	33.9(33, 35.2)	0.12
Median (IQR) age at MRI/MRS (d)	7(5, 8.5)	10.2 (5, 12)	0.24
Sex (M:F)	5:6	8:9	0.79
Sarnat NE stage mild (n)	0	6	<b>0.01</b>
Sarnat NE moderate (n)	9	7	0.71
Sarnat NE severe (n)	2	4	0.45
Infants with unfavourable outcome (n)	3	3	0.8

**Table 5.1:** Clinical characteristics (IQR - interquartile range)

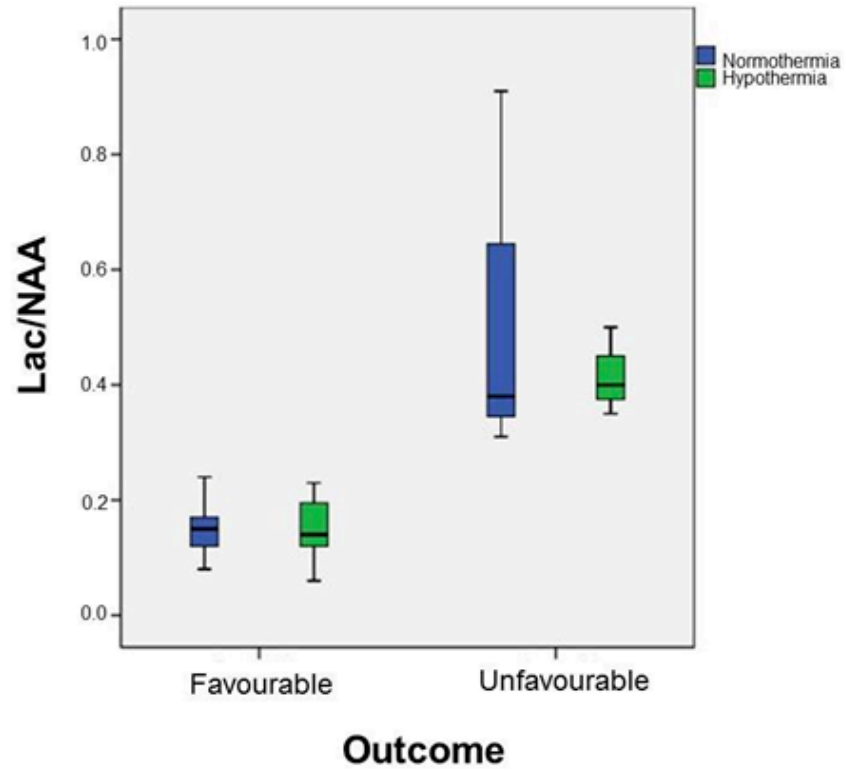
	Lac/Naa		Lac/Cr		MRI abnormalities		Abnormal PLIC	
	Normothermia	Hypothermia	Normothermia	Hypothermia	Normothermia	Hypothermia	Normothermia	Hypothermia
<b>Sensitivity</b> <b>(95%CI):</b>	100 (55 – 100)	100 (57 - 100)	100 (48 – 100)	67 ( 27-67)	100 (48 – 100)	66 (24 – 93)	66 (22 – 93)	66 (24 – 93)
<b>Specificity</b> <b>(95%CI):</b>	100 (90 – 100)	100 (84 – 100)	71 (60 – 71)	100 (85– 100)	71 (60 – 71)	75 (59 – 85)	78 (69 – 84)	75 (59 – 85)
<b>PPV (95%CI):</b>	100 (55 – 100)	100 (57 – 100)	42 (20 – 42)	100 (40 – 100)	42 (20 – 42)	50 (18 – 70)	40 (13 – 56)	50 (18 – 70)
<b>NPV (95%CI):</b>	100 (90 – 100)	100 (84 – 100)	100 (84 – 100)	89 (75 – 89)	100 (84 – 100)	85 (67 – 97)	91 (80 – 98)	85 (67 – 97)
<b>DOR</b> <b>(95%CI):</b>	168 (4.8 – 6169)	96 (2.6 – 3590)	15 (0.99 – 188)	32 (1.3 – 640)	15 (0.99 – 188)	6 (0.45 – 75)	7.3 (0.6 – 76)	6 (0.45 – 75)
<b>AUC (SE) :</b>	0.97 (0.03)	1.00 (0.00)	1.00 (0.00)	0.97 (0.04)	0.81 (0.09)	0.75 (0.19)	0.72 (0.17)	0.70 (0.18)

**Table 5.2: MRI and MRS sensitivities and specificities for unfavourable outcome and predictive values, diagnostic odds ratios and AUC.**

	Unadjusted OR (95% CI)	Probability (p)	Adjusted OR (95% CI)	Probability (p)
<b>Lac/NAA</b>	15 (3.5, 62)	<b>0.01</b>	2 (0.97, 4.2)	<b>0.05</b>
<b>Lac/Cr</b>	6.3 (4, 83)	<b>0.01</b>	1.9 (0.9, 9.1)	<b>0.05</b>
<b>Abnormal PLIC</b>	0.23 (0.03, 1.4)	0.11	1.3	0.99
<b>MRI abnormalities</b>	0.16 (0.02, 1.0)	<b>0.05</b>	1.3	0.99

**Table 5.3:** Biomarker odds ratios and significances from logistic regression analysis





**Figure 5.4:** Thalamic Lac/NAA for favourable and unfavourable outcome (mean and 95% CI).

Case ID	Study Group	Cognitive scale	Language scale	Motor scale	Neurology Examination
1	N	106	95	90	Normal
2	N	70	74	65	Global delay and Microcephaly
3	N	95	121	103	Normal
4	N	90	74	110	Normal
5	N	115	138	103	Normal
6	N	110	74	86	Normal
7	N	100	109	94	Normal
8	N	102	95	90	Normal
9	N	115	118	118	Normal
10	N	100	127	112	Normal
11	N	110	102	100	Normal
12	N	80	72	65	Hemiplegic CP
13	N	95	83	75	Normal
14	N	112	95	95	Normal
15	N	95	91	86	Normal
16	N	70	79	69	Global delay and Microcephaly
17	N	100	90	92	Normal
18	H	140	112	110	Normal
19	H	105	103	136	Normal
20	H	60	56	58	Spastic CP with visual problems
21	H	120	97	100	Normal
22	H	100	103	97	Normal
23	H	100	100	91	Normal
24	H	80	83	69	Deafness and motor delay
25	H	95	98	110	Normal
26	H	78	70	60	Hemiplegic CP
27	H	110	103	88	Normal
28	H	105	91	112	Normal

**Table 5.4:** Outcome data as measured at 18 months of age. The Bailey 3 scores and neurological examination findings of all infants in both study groups are shown here. N - Normothermia, H – Hypothermia.

## Discussion

This paper reports the accuracies of cerebral MRI and MRS biomarkers for predicting adverse outcome in infants who received therapeutic hypothermia for NE. Consistent with the conclusion of our recent systematic review (Thayyil, Chandrasekaran et al. 2010), early deep gray matter Lac/NAA was the most accurate predictor of long term adverse neurological outcome following NE and Lac/NAA prediction accuracy was unaltered by preceding therapeutic hypothermia. The discriminatory power of qualitative reporting of conventional MRI and abnormal PLIC was poor.

To the best of our knowledge, this is the first study to report the prognostic accuracies of cerebral MRI and MRS biomarkers in encephalopathic babies who have had therapeutic hypothermia. An extensive recent meta-analysis of cerebral MRI and MRS biomarkers in babies who had no hypothermic intervention for NE concluded that the most accurate outcome predictor was deep gray matter Lac/NAA (Thayyil, Chandrasekaran et al. 2010). Conventional MRI and PLIC abnormality were less prognostic. The results presented in this study from babies who had or did not have prior hypothermic treatment are consistent with these findings. Thalamic Lac/NAA had 100% specificity in both uncooled and hypothermia pre-treated babies, while this ratio had 95% specificity in the meta-analysis in uncooled babies.

Conventional MRI had 71% specificity in uncooled babies and 75% in hypothermia pre-treated babies, while this modality had 51% specificity in the meta-analysis in uncooled babies. The diagnostic accuracies of conventional MRI in our study (uncooled 0.81, hypothermia pre-treated 0.75) were similar to those in a recently published large randomised control study (Rutherford, Ramenghi et al. 2010). However, the diagnostic accuracy of thalamic Lac/NAA was always better.

It is essential to understand Lac and NAA time-dependences after hypoxia-ischemia, and particularly during therapeutic hypothermia. There are characteristic spectrum changes in infants with NE, especially Lac elevation (conventionally interpreted as a result of brain ischemia and hypoxia), and a fall in NAA (suggestive of neuronal injury). However NAA falls slower than Lac rises (Petroff, Ogino et al. 1988, Graham, Blamire et al. 1993). From animal studies, we know that cerebral Lac rises during hypoxia-ischemia, but normalises after resuscitation and reperfusion (Lei and Peeling 1998). However, there is a further delayed Lac rise several hours later possibly due to renewed Lac production or mitochondrial failure (Penrice, Lorek et al. 1997). This rise may persist for considerable time (Hanrahan, Cox et al. 1998). In global ischemia in gerbils, decreased brain NAA correlated with neuronal loss and remaining neuronal density (Sager, Topp et al. 2001) In our piglet transient hypoxia-ischemia model, the extents of the rise in brain Lac and NAA fall over the first 48 h after hypoxia-ischemia are strongly associated with nuclear DNA fragmentation in dying cells detected with – terminal Transferase d-UTP Nick End-Labeling (TUNEL) histochemistry (Chandrasekaran M 2010).

Furthermore, in brain cortex and thalamus, cellular metabolism is temperature dependent (Mendelowitsch, Mergner et al. 1998, Jia, Yue et al. 2008). In our piglet hypoxia-ischemia model, we monitored cerebral metabolism with MRS every 6 h during therapeutic hypothermia and found lessened Lac rise during hypothermia (unpublished data). However, high cortical Lac levels were observed in normal Sprague–Dawley rats during mild hypothermia (Chan, Chow et al. 2010). Hence, the predictive value of Lac during hypothermia is uncertain.

An advantage of this study was that the MRI scanners and protocols were the same throughout: however, there were limitations. Firstly this is a prospective observational study conducted in a single neonatal unit before and after introduction of rescue hypothermic neuroprotection as standard care, rather than a randomised control trial.

As a result, the NE severity of uncooled babies was dissimilar to that of those who were cooled. However, this study's intention was to examine the prognostic accuracies of MRI and MRS biomarkers in a cohort resembling very closely that expected clinically rather than to examine the treatment effects of hypothermia.

Secondly, the presented metabolite ratio results are relevant only when exactly the same protocols and scanners are used. Hence, caution must be exercised when using cerebral MRI and MRS biomarkers as surrogate outcome measures in multicentre studies. Metabolite ratio variance increase of about 10% has been reported in multicentre studies using different scanners. Hence, our data may be unusable for sample size calculation for multicentre trials of neuroprotective therapies employing MRI or MRS as a surrogate outcome measure.

Finally, as with all previous single-centre studies examining MRI prognostic accuracy, our sample size is small and this is reflected in the wide estimate confidence intervals. Thus our findings need confirmation in larger cohorts.

## **Conclusion**

Prior therapeutic hypothermia did not alter thalamic Lac/NAA or Lac/Cr outcome prediction accuracy. Our data show that after moderate therapeutic hypothermia for NE in the early neonatal period (age < 2 weeks) thalamic Lac/NAA remains a robust biomarker predictive of outcome. MRS metabolite ratios may be useful surrogate biomarkers in future trials of neuroprotective therapies in such infants.

## **Chapter 6 - Conclusion and further research**

### **The predictive value of Early MRS biomarkers (EBMs), acquired around 4h after HI, in the piglet asphyxia model**

This study demonstrated that EBMs, acquired between 2h and 4h after a hypoxic-ischemic insult, predict the evolution of the energy disturbances on MRS and histopathological outcome at 48 hours after HI. Specifically, thalamic Lac/NAA and Lac/Cr AUC showed strong association with the outcome in both Xe/HT and MIA experimental series.

From our study, there was a very close correlation between Lac/NAA (acquired early) and TUNEL – positive cells. This important finding can be of enormous use to research and clinical areas. As several neuroprotective drugs still need to be tested in animal experiments, it is essential to standardize the HI insult severity and reduce the variability in the outcome. EBMs, particularly Lac/NAA, could play a significant role in reducing the variability. If we could predict the severity of the outcome by EBMs, acquired as early as 2-4h after HI (within the therapeutic window), appropriate candidates could be selected for neuroprotective intervention in clinical studies. It can be difficult and challenging to identify newborn infants who could potentially benefit from neuroprotection because of the evolving clinical presentation. Even though the therapeutic window for neuroprotective intervention is brief, it is not impossible to acquire early MRS biomarkers within the required time. Thus EBMs can be used to identify the ideal candidates in order to reduce costs and time spent on these trials.

Approximately, 25% of encephalopathic infants die in the neonatal period due to the severity of the injury and a clinical redirection of care. Clinicians use wide range of clinical, electrophysiological (aEEG) and neuroimaging techniques to assess the severity of encephalopathy. Even though, there is some evidence for a early aEEG to prognosticate long term outcome (Toet, Hellstrom-Westas et al. 1999), the value of early MRI to predict long term outcome is poor (Thayyil, Chandrasekaran et al.

2010). It is essential that we offer better counselling to parents regarding outcomes and to avoid offering false hope to parents. EBMs could play a significant role in decision making for these infants.

### **The predictive value of late MRS biomarkers, acquired around 48h after HI, in the piglet asphyxia model**

This study demonstrated that MRS biomarkers, acquired at 48 hours after HI, predict the histological outcome in untreated, control animals. This study also has shown that neuroprotective intervention did not affect the predictive ability of these biomarkers. More specifically, thalamic and WM Lac/NAA and Lac/Cr showed good outcome prediction in control animals as well as in animals who received neuroprotective intervention in both Xe/HI and MIA experimental series.

This is an important finding as these MRS biomarkers can be used as surrogate end point in future neuroprotective trials. Despite therapeutic hypothermia, up to 50% of newborn infants still have abnormal neurological outcome (Edwards, Brocklehurst et al. 2010). Currently research is being focused on developing and evaluating several neuroprotective adjuncts (Kelen and Robertson 2010). While evaluating these neuroprotective drugs, these MRS biomarkers can provide rapid results as outcome measures and can help to speed up the translation of these drugs to clinical research.

### **The predictive value of MRS biomarkers in newborn babies with HI - Clinical translation of MRS biomarkers**

This study has shown that thalamic Lac/NAA acquired between 4 and 14 days of age, predicts long term neurodevelopmental outcome at 18 months of age. This study also demonstrated that prior therapeutic hypothermia did not alter thalamic Lac/NAA outcome prediction accuracy.



This novel finding is a key in validating MRS biomarkers as a clinical outcome measure. These studies prove that MRS can be trusted as a bridging biomarker. In clinical neuroprotection trials, MRS should be used as a surrogate end point so that the results can be obtained earlier to save time and money. This also helps in more rapid clinical application of effective neuroprotectants and saving more infants from disability.

## **Further research**

### Optimisation of MRS acquisition

The presented metabolite ratio results are relevant only when exactly the same protocols and scanners are used. Hence, caution must be exercised when using cerebral MRI and MRS biomarkers as surrogate outcome measures in multicentre studies. Also Lac detection depends on TE of the pulse sequence used. Use of 3T is increasing, and optimization and standardization of acquisition methods are needed.

### Absolute quantification of metabolites

It is well known that NAA is a neuronal marker. In global ischaemia, decreases in brain NAA are closely correlated neuronal loss and remaining neuronal density (Sager, Topp et al. 2001). Also on secondary analysis of the systematic review (Thayyil, Chandrasekaran et al. 2010), the predictive ability of absolute quantification of NAA concentration was promising, observed in 2 studies. NAA concentrations may therefore be important for monitoring neuroprotective interventions in the newborn with perinatal asphyxial encephalopathy. A large prospective international study evaluating cerebral MRI biomarkers including absolute quantification, as surrogate outcome measures is currently in progress (MARBLE study– Magnetic Resonance Biomarkers in Neonatal Encephalopathy; Clinical Trials No: NCT01309711). Future animal studies may consider evaluating the relationship between absolute concentrations of metabolites with Tunel positive cell

death and microglial ramification.

#### Long term Follow up

The predictive value of MRS biomarkers is based on 18 month – neurodevelopmental outcome from the current study. However it is essential to follow these infants for longer duration, at least up to 5 years to evaluate the full neurological impairment. This will ensure the further validation of these biomarkers.

#### Predictive value of WM MRS metabolite ratios

From the results of animal studies as mentioned in previous chapters, WM Lac/NAA and other WM metabolite ratios also had good correlation with histological outcome. However the predictive value of WM MRS metabolite ratios is not known in newborn infants. This should be evaluated in future studies as this might provide great insights into cognitive outcome.

#### The course of microglial activation after neuroprotective intervention

As many studies have shown the activation, proliferation and de-ramification of microglia following HI, little is known about the microglial course following neuroprotective intervention like hypothermia. Although we noticed the preservation of ramification to some extent, it is difficult to explain the poor correlation to MRS metabolite ratios following neuroprotection. Further studies are needed to analyse the course of microglia following neuroprotective intervention.

#### MRS metabolite changes during hypothermia

This study has shown that hypothermia does not affect the predictive accuracy of thalamic Lac/NAA and Lac/Cr when acquired after hypothermia treatment. The influence of treatments on lactate generation following hypoxia-ischaemia is unknown but would be of interest.

A limitation in the translation of experimental work to the clinic is that, unlike the experimental set-up, in infants with NE, the precise timing of the hypoxia-ischaemia is unknown. This study is reassuring, however, as it shows that MRS biomarkers, particularly thalamic Lac/NA, are predictive both early (2-4h) and late (48h), making knowledge of the timing of the insult less vital.

## Appendix

### A – Publications derived from this work

1. *Xenon augmented hypothermia reduces early lactate/N-acetylaspartate and cell death in perinatal asphyxia.* Faulkner S, Bainbridge A, Kato T, **Chandrasekaran M**, Kapetanakis AB, Hristova M, Liu M, Evans S, De Vita E, Kelen D, Sanders RD, Edwards AD, Maze M, Cady EB, Raivich G, Robertson NJ. *Ann Neurol.* 2011 Jul;70(1):133-50. (attached)
2. *Methyl-isobutyl Amiloride Reduces Early Brain Lactate/N-Acetylaspartate and Cell Death in a Piglet Perinatal Asphyxia Model* Nicola J Robertson, Takenori Kato, Alan Bainbridge, **Manigandan Chandrasekaran**, Osuke Iwata, Andrew Kapetanakis, Enrico DeVita , Stuart Faulkner, Jeanie Cheong, Sachiko Iwata, Mariya Hristova, Ernest Cady, Gennadij Raivich – *J Neurochem*, 2013 Mar;124(5):645-57. (attached)
3. *Prognostic Accuracy of Cerebral Proton Magnetic Resonance Spectroscopy after Therapeutic Hypothermia for Neonatal Encephalopathy.* **Manigandan Chandrasekaran**, Sudhin Thayyil , Alan Bainbridge , Giles Kendall , Angela Huertas-Ceballos , Roxanna Gunny , Neil Marlow , Ernest Cady, Gennadij Raivich , Nicola J Robertson. - under preparation
4. *Early Cerebral MRS Biomarkers in a Piglet Model of Perinatal Asphyxia: Prognostic Value in Neuroprotection Studies* **Manigandan Chandrasekaran MRCPCH**, Stuart Faulkner, Takenori Kato , Alan Bainbridge , Ernest Cady, Nicola J Robertson , Gennadij Raivich – under preparation

### B – Publications related to this work

1. *Cerebral magnetic resonance biomarkers in neonatal encephalopathy: a meta-analysis.* Thayyil S, **Chandrasekaran M**, Taylor A, Bainbridge A, Cady EB, Chong WK, Murad S, Omar RZ, Robertson NJ. *Pediatrics.* 2010 Feb;125(2):e382-95.

### C – Presentations in international and national conferences

1. *Xenon combined with hypothermia reduces cerebral lactate/Cr on 1H MRS, markers of cell death and phagocytosis at 48 Hours in a perinatal asphyxia piglet model.* **Chandrasekaran M**, Faulkner S, Kelen D, Kato T, Thayyil S, Bainbridge A, Cady E, Raivich G, Robertson N. Pediatric Academic Society meeting 2632.7 May 2010
2. *Amiloride is neuroprotective in the piglet neonatal hypoxic ischaemic encephalopathy model.* **M Chandrasekaran**, T Kato, O Iwata, A Bainbridge, E Cady, G Raivich, N J Robertson. *Arch Dis Child Fetal Neonatal Ed* 2011;96:Fa27 2011.300164.37. Perinatal Medicine, Harrogate, UK, June 2011.

3. *Thalamic 1H MRS Lactate/NAA Predicts Neurodevelopmental Outcome in Both Cooled and Non-Cooled Infants Following Perinatal Asphyxia.* **Manigandan Chandrasekaran**, Sudhin Thayyil, Alan Bainbridge, Giles Kendall, Angela Huertas-Ceballos, Roxanna Gunny, Neil Marlow, Ernst Cady, Nicola J. Robertson. Pediatric academic society meeting 2910.123, May 2011.
4. *Prognostic Accuracy of Cerebral Proton Magnetic Resonance Spectroscopy after Therapeutic Hypothermia for Neonatal Encephalopathy.* **Manigandan Chandrasekaran**, Sudhin Thayyil , Alan Bainbridge , Giles Kendall , Angela Huertas-Ceballos , Roxanna Gunny , Neil Marlow , Ernest Cady, Gennadij Raivich , Nicola J Robertson. – European society of paediatric research, Newcastle, UK, 14/10/2011.

**Table 1**

Variables	N (n=9)	Xe (n=8)	Hypo (n=8)	Xe+Hypo (n=7)
Postnatal age (hr)	23.3 (0.9)	23.3 (0.7)	23.6 (0.7)	23.3 (0.9)
Body weight (g)	1688 (221)	1719 (365)	1694 (253)	1763 (107)
Insult severity (hr×10 <sup>-2</sup> )	10.4 (2.5)	11.5 (2.4)	11.7 (4.1)	11.5 (2.4)
<b>HR</b> (bpm) Baseline	163 (15)	161 (16)	160 (17)	167 (18)
End of insult	188 (22) <sup>a</sup>	169 (16)	169 (18)	176 (18)
2-26 hr after time zero	161 (19)	165 (23)	133 (26) <sup>d,e</sup>	134 (24) <sup>d,e</sup>
48 hr after time zero	129 (12)a	135 (20)a	153 (17)cd	144 (18)
<b>MABP</b> (mmHg)Baseline	46 (4)	43 (5)	41 (6)	44 (6)
End of insult	53 (5)a	47 (4)	47 (4)	46 (7)
2-26 hr after time zero	48 (6)	43 (4) <sup>d</sup>	40 (6) <sup>d</sup>	40 (7) <sup>d</sup>
26-48 hr after time zero	46 (8)	47 (5)	40 (3)	45 (5)
<b>T rectal</b> (°C) Baseline	38.7 (0.4)	39.0 (0.1)	38.8 (0.2)	38.8 (0.4)
End of insult	38.6 (0.3)	38.5 (0.4)	38.5 (0.2)	38.5 (0.3)
6-26 hours after time zero	38.6 (0.3)	38.7 (0.3)	33.3 (0.3) <sup>a,d,f</sup>	33.4 (0.4) <sup>a,d,f</sup>
48 hr after time zero	38.5 (0.2)	38.5 (0.3)	38.6 (0.4)	38.9 (0.2)
<b>PaO2</b> (kPa) Baseline	9.7 (1.2)	11.4 (1.8)	10.9(1.2)	10.6 (1.9)
nadir of the insult	2.9 (0.3)a	4.3 (1.0)ad	2.7 (0.5)a	6.0 (1.2)ade
24 hr after time zero	13.3 (1.4)	13.0 (1.2)	12.1 (1.2)	14.8 (1.9)
48 hr after time zero	13.2 (3.4)	18.9 (12.4)	15.1 (4.5)	12.0 (3.5)
<b>PaCO2</b> (kPa) Baseline	5.1 (0.9)	6.2 (0.4)	5.9 (1.6)	6.0 (1.3)
nadir of the insult	5.3 (0.7)	5.0 (0.3)a	4.9 (1.2)	4.6 (1.1)a
24 hr after time zero	4.7 (2.4)	5.2 (1.9)	4.3 (3.3)	5.6 (1.9)

48 hr after time zero	5.5 (1.9)	5.4 (2.2)	3.8 (3.0)	5.8 (1.7)
<b>pH</b> Baseline	7.52 (0.06)	7.46 (0.04)	7.49 (0.09)	7.48 (0.07)
nadir of the insult	7.42 (0.10) <sup>a</sup>	7.48 (0.05)	7.47 (0.11)	7.49 (0.07)
24 hr after time zero	7.47 (0.11)	7.50 (0.15)	7.44 (0.12)	7.45 (0.11)
48 hr after time zero	7.43 (0.13)	7.44 (0.11)	7.46 (0.11)	7.36 (0.07)
<b>BE (mmol/L)</b> Baseline	7.2 (2.7)	8.9 (2.7)	8.6 (2.5)	8.3 (2.4)
nadir of the insult	1.4 (5.9) <sup>a</sup>	4.2 (3.4) <sup>a</sup>	4.2 (4.2) <sup>a</sup>	4.2 (3.1) <sup>a</sup>
24 hr after time zero	6.2 (3.5)	6.7 (5.3)	6.4 (6.3)	5.4 (1.1)
48 hr after time zero	1.8 (2.9) <sup>ac</sup>	3.6 (4.6) <sup>a</sup>	2.7 (2.8) <sup>ac</sup>	1.6 (2.5) <sup>a</sup>
<b>Lactate(mmol/L)</b> Baseline	3.2 (1.9)	2.7 (0.6)	2.5 (0.6)	2.6 (0.7)
nadir of the insult	7.4 (2.4) <sup>a</sup>	6.0 (1.4) <sup>a</sup>	5.5 (1.9) <sup>a</sup>	6.1 (0.8) <sup>a</sup>
24 hr after time zero	1.4 (0.4)	1.9 (0.6)	0.9 (0.3)	1.1 (0.4)
48 hr after time zero	1.7 (0.7)	1.7 (0.3)	1.4 (0.4)	1.4 (0.5)
<b>*glucose(mmol/L)</b> Baseline	7.2 (1.0)	7.3 (0.8)	6.9 (1.1)	7.2 (1.7)
nadir of the insult	9.3 (1.9)	8.6 (1.4)	8.6 (1.8)	10.0 (2.2)
24 hr after time zero	4.5 (1.3)	4.6 (1.8)	5.2 (0.7)	9.7 (4.6)
48 hr after time zero	5.1 (0.9)	4.7 (1.1)	4.0 (1.1)	4.9 (0.2)

**Table 1.** Physiological variables for piglets in each group of Xe/HT study. Mean (SD) values are presented for each group. One-way ANOVA and post hoc analysis was carried out on comparisons between groups with Bonferroni correction for repeated measures. Time zero was set at the start of resuscitation after insult (HI). Insult severity index was defined as the time integral of the acute energy depletion (AED) during HI and for 60mins of resuscitation. a:  $p < 0.05$  within group comparisons vs baseline; b:  $p < 0.01$ ; c:  $p < 0.05$  within group comparisons vs 24h after time zero; d:  $p < 0.01$  vs normothermia at the same time point or during the same time period; e:  $p < 0.01$  vs Xe at the same time point or during the same time period; f:  $p < 0.05$  vs Xe at the same time point. \* insufficient data points to complete statistical analysis. Permission to reproduce this table has been granted by John Wiley and Sons.

**Table 2**

Variables	Time	MIA Group Mean (SD)	Placebo Group Mean (SD)	p value (MIA vs Placebo)
Number of piglets		n=9	n=9	
Postnatal age (h)		21.9 (13-26.5)	23.4 (21.5-28)	0.47
Bodyweight (g)		1870 (220)	1830 (190)	0.74
Insult severity (10 <sup>-2</sup> h)		11.6 (1.4)	12.9 (3.0)	0.25
Rectal Temperature (°C)	Baseline	38.2 (0.5)	38.4 (0.4)	0.32
	HI end	38.7 (0.7)	38.6 (0.7)	0.67
	2.5-26h	<b>38.7 (0.5)*</b>	38.6 (0.2)	0.39
	26.5-48h	38.5 (0.3)	38.5 (0.2)	0.91
Heart rate (min <sup>-1</sup> )	Baseline	177 (27)	195 (19)	0.13
	HI end	<b>152 (27)*</b>	194 (21)	<b>0.002</b>
	2.5-26h	<b>147 (15)*</b>	183 (13)	<b>&lt;0.001</b>
	26.5-48h	<b>130 (15)*</b>	<b>162 (13)*</b>	<b>&lt;0.001</b>
MABP (mmHg)	Baseline	47 (9)	45 (6)	0.67
	HI end	45 (6)	44 (4)	0.60
	2.5-26h	46 (5)	45 (6)	0.57
	26.5-48h	48 (7)	46 (7)	0.44
Isoflurane (%)	Baseline	2.7 (0.5)	2.7 (0.3)	0.95
	HI end	2.1 (0.0)	-	(°)
	2.5-26h	2.4 (0.2)	2.4 (0.5)	0.77
	26.5-48h	2.3 (0.3)	2.1 (0.6)	0.30
FiO <sub>2</sub> (%)	Baseline	32 (6)	33 (3)	0.71
	HI end	35 (6)	52 (0)	
	2.5-26h	33 (17)	35 (9)	0.75
	26.5-48h	35 (4)	51 (13)	0.07
Blood pH	Baseline	7.46 (0.08)	7.46 (0.08)	0.94
	HI end	7.44 (0.01)	7.39 (0.01)	(°)
	2.5-26h	7.46 (0.08)	7.49 (0.10)	0.54
	26.5-48h	7.45 (0.07)	7.38 (0.20)	0.36
PaCO <sub>2</sub> (kPa)	Baseline	6.0 (1.3)	5.5 (1.4)	0.48
	HI end	5.9 (0.4)	6.8 (0.0)	(°)
	2.5-26h	5.6 (1.2)	5.1 (1.4)	0.47
	26.5-48h	5.3 (1.3)	5.6 (1.2)	0.62
PaO <sub>2</sub> (kPa)	Baseline	8.8 (1.6)	9.2 (1.7)	0.62
	HI end	10.5 (3.0)	13.2 (1.6)	(°)
	2.5-26h	<b>13.2 (3.7)*</b>	<b>13.4 (3.1)*</b>	0.89
	26.5-48h	<b>15.0 (1.7)*</b>	<b>13.8 (2.1)*</b>	0.26
Base excess (mmol/L)	Baseline	6.9 (1.9)	4.3 (2.5)	<b>0.02</b>
	HI end	5.5 (0.7)	5.0 (0.4)	(°)
	2.5-26h	4.9 (3.1)	4.7 (5.0)	0.90
	26.5-48h	2.4 (3.2)	2.1 (4.8)	0.87
Blood Sodium	Baseline	141 (6)	139 (3)	0.28



(mmol/L)				
	HI end	141 (11)	139 (0)	(°)
	2.5-26h	<b>136 (4)*</b>	<b>135 (3)*</b>	0.52
	26.5-48h I	<b>133 (9)*</b>	<b>133 (6)*</b>	0.89
Blood Potassium (mmol/L)	Baseline	4.0 (0.8)	4.1 (1.0)	0.81
	HI end	3.8 (1.8)	4.6 (0.6)	(°)
	2.5-26h	<b>5.1 (1.4)*</b>	<b>5.1 (1.0)*</b>	0.99
	26.5-48h	<b>5.6 (1.3)*</b>	5.0 (1.5)	0.39
Blood Lactate (mmol/L)	Baseline	2.5 (0.9)	3.6 (1.4)	0.06
	HI end	2.5 (1.2)	2.5 (0.0)	(°)
	2.5-26h	<b>1.7 (1.0)*</b>	<b>1.9 (0.7)*</b>	0.66
	26.5-48h	<b>1.5 (0.5)*</b>	<b>1.3 (0.7)*</b>	0.55

**Table 2.** Physiological variables for piglets in the MIA study. Measurements were made at baseline and during 3 time periods: 0.5-2h post HI (HI end), 2.5-26h post HI, and 26.5-48h post HI. Each variable was averaged over each time period for each animal. Intergroup comparisons used the unpaired t test (right column). \* $p < 0.05$  against baseline value in the same group (MIA treated or placebo) using paired t-test. ° Insufficient data for statistical testing. There were no differences between groups at baseline apart from a slightly more alkaline blood pH in the MIA group. There were no physiological and serum electrolyte or biochemical differences between groups following HI apart from a lower heart rate in the MIA group. Permission to reproduce this figure has been granted by John Wiley and Sons.

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